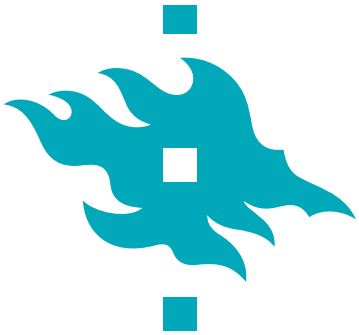

DISSERTATIONES SCHOLAE DOCTORALIS AD SANITATEM INVESTIGANDAM
UNIVERSITATIS HELSINKIENSIS

ANDRII BUGAI

REGULATION OF POL II TRANSCRIPTIONAL RESPONSE TO DNA DAMAGE BY RBM7 AND P-TEFb

MEDICUM
DEPARTMENT OF BIOCHEMISTRY AND DEVELOPMENTAL BIOLOGY
FACULTY OF MEDICINE
DOCTORAL PROGRAMME IN BIOMEDICINE
UNIVERSITY OF HELSINKI

Doctoral Programme in Biomedicine
Department of Biochemistry and Developmental Biology
Faculty of Medicine
University of Helsinki

REGULATION OF POL II TRANSCRIPTIONAL RESPONSE TO DNA DAMAGE BY RBM7 AND P-TEFB

Andrii Bugai

ACADEMIC DISSERTATION

To be presented for public discussion with the permission of the Faculty Medicine of the University of Helsinki, in Lecture Room 107, Athena, Siltavuorenpenger 3A, Helsinki on the 22nd of January 2020, at 12 noon.

Helsinki 2020

ISBN 978-951-51-5767-6 (pbk)
ISBN 978-951-51-5768-3 (PDF)
ISSN 2342-3161 (pbk)
ISSN 2342-317X (PDF).
http://ethesis.helsinki.fi

Cover layout by Anita Tienhaara

Helsinki 2020

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To my family

1 ABSTRACT

DNA damage response (DDR) is a cascade of events within the cells, which is initiated by DNA lesions and results in DNA repair and cell survival. Alternatively, DDR could lead to apoptosis - elimination of cells, in which genetic integrity is impossible to restore. Despite the commonly accepted paradigm that RNA synthesis is shut down following DNA damage, recent studies suggest that transcription of DDR genes is activated. The aim of my dissertation is to shed new light on the molecular mechanisms of the transcriptional response to DNA damage.

Gene transcription is the process of transfer of genetic information from DNA to RNA. There are three major steps of transcription: initiation, elongation, and termination. Pausing in early elongation is a key control point of RNA polymerase II (Pol II)-mediated transcription. Negative transcription elongation factors (N-TEFs) interact with Pol II to mediate promoter-proximal pausing. Cyclin-dependent kinase 9 (CDK9) of the positive transcription elongation factor b (P-TEFb) phosphorylates N-TEFs and the C-terminal domain (CTD) of Pol II to resume transcription of paused genes. Small nuclear ribonucleoprotein complex containing 7SK RNA (7SK snRNP) regulates P-TEFb, offering a possibility for the rapid transcription of DDR genes following genotoxic stress.

Here I provided new insight into the molecular mechanism of the transcriptional response to DNA damage. Using high-throughput protein-RNA interactome mapping by UV crosslinking and immunoprecipitation (iCLIP), nascent RNA sequencing, quantitative PCR, and RNA interference experiments I showed that, following genotoxic stress, RNA-binding motif protein 7 (RBM7) stimulated Pol II pause release by activating the P-TEFb via its release from the inhibitory 7SK snRNP. This was mediated by activation of p38 MAPK, which triggered enhanced binding of RBM7 with core subunits of 7SK snRNP. In turn, P-TEFb relocated to chromatin to induce transcription of short units, including key DDR genes and multiple classes of non-coding RNAs (ncRNA). Inhibition of the CDK9 subunit of P-TEFb or depletion of RBM7 provoked cellular hypersensitivity to DNA-damage-inducing agents via activation of apoptosis. In sum, my work suggests that RBM7 controls transcriptional response to DNA damage through P-TEFb. Moreover, it uncovers the importance of stress-dependent stimulation of the Pol II pause release, which enables a pro-survival transcriptional response that is crucial for cell fate upon genotoxic insult.

2 АНОТАЦІЯ

Регуляція експресії генів є фундаментальною ознакою всіх живих істот. Зокрема, процес передачі генетичної інформації, що зберігається в ДНК, в молекули матричних РНК, транскрипція за допомогою РНК-полімерази II, є критичним елементом регуляції активності генів. Зпоміж механізмів, що регулюють транскрипцію важливих високозарегульованих генів у багатоклітинних організмах, саме регуляція на стадії елонгації транскрипції є ключовою. РНК-полімераза II, починаючи транскрипцію з промоторів таких генів, призупиняється незабаром після початку синтезу РНК в очікуванні на додатковий сигнал для їх повноцінної експрессії. Позитивний фактор елонгації транскрипції Б (P-TEFb), що представляє собою гетеродимер циклін-залежної кінази 9 (CDK9) та цикліну T, є критичним регулятором продуктивної транскрипції на цій стадії, фосфорилюючи С-кінцевий домен головної субодиниці РНК-полімерази. Зважаючи на ключову роль P-TEFb для активації експерссії генів його функція в свою чергу регулюється за допомогою 7SK малого ядерного рибонуклеопротеїду (7SK snRNP), що має властивість інгібітора кіназної активности CDK9, а також молекулярних факторів, які вивільняють P-TEFb з 7SK snRNP та доставляють до промоторів генів.

У моїй дисертаційній роботі я описав новий механізм регуляції транскрипції, що працює в умовах пошкодження ДНК клітин людини, за допомогою вивільнення P-TEFb з 7SK snRNP, опосередкованим РНК зв'язуваючим мотивом 7 (RBM7). Незважаючи на загальну парадигму у галузі щодо інгібіції транскрипції внаслідок пошкодження ДНК, поточна робота вказує що активація елонгації специфічних генів за допомогою P-TEFb є необхідною для виживання клітини в таких умовах. Результати, отримані в ході цієї роботи, проливають світло на новий елемент стійкості клітин людини до агентів, що пошкоджують ДНК, а також можливі шляхи подолання цієї стійкості для терапевтичних потреб.

3 CONTENTS

4 ORIGINAL PUBLICATION

This thesis is based on the following publication:

I. **Bugai A,** Quaresma AJC, Friedel CC, Lenasi T, Düster R, Sibley CR, Fujinaga K, Kukanja P, Hennig T, Blasius M, Geyer M, Ule J, Dölken L, Barboric M. (2019) P-TEFb activation by RBM7 shapes a pro-survival transcriptional response to genotoxic stress. *Mol Cell,* 74, 254-267.

The original publication is referenced in the text as "I", reprinted in original form with the permission of the publisher.

As the first author on the publication, Andrii Bugai contributed to the planning and performing experiments, analyzing the experimental data, and writing the manuscript as specified in the report on the author's contribution to the article which is enclosed as an appendix of I.

Unpublished results are also presented.

5 ABBREVIATIONS

4-NQO	4-nitroquinoline 1-oxide
4sU-seq	4sU-RNA purification followed by sequencing
5-FU	5-fluorouracil
7SK	7SK small nuclear RNA
7SK snRNP	7SK small nuclear ribonucleoprotein
Akt	RAC-alpha serine/threonine-protein kinase
ATM	Ataxia telangiectasia serine/threonine kinase
ATR	Ataxia telangiectasia and Rad3-related protein serine/threonine kinase
CDK7	Cyclin-dependent kinase 7
CDK9	Cyclin-dependent kinase 9
CDK12	Cyclin-dependent kinase 12
CLIP	Crosslinking Immunoprecipitation
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation followed by sequencing
CS	Cockayne syndrome
CTD	C-terminal domain
CTG	CellTiter Glo cell survival assay
CTxG	CellTox Green cytotoxicity assay
CYCT	Cyclin T
CYCK	Cyclin K
DMEM	Dulbecco's modified Eagle medium
DDR	DNA damage response
DDX21	DExD-box helicase 21
DRB	5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside
DSIF	DRB sensitivity inducing factor
ELL1/2/3	Eleven-nineteen Lys-rich Leukemia member
ERK	Extracellular signal-regulated kinase
eRNA	enhancer RNA
FBS	Fetal bovine serum
JMJD6	Jumonji domain containing 6, arginine demethylase and lysine hydroxylase
GRO-seq	Global nuclear run-on following by sequencing
GTF	General transcription factor
IP	Immunoprecipitation
qPCR	Quantitative polymerase chain reaction
HEXIM1	Hexamethylene bisacetamide-induced protein 1
HIV	Human immunodeficiency virus

hnRNP	Heterogeneous nuclear ribonucleoprotein
HTLV	Human T-lymphotropic virus
kbp	kilobase pair
LARP7	La-related protein 7
lncRNA	Long non-coding RNA
MAPK	Mitogen-activated protein kinase
MePCE	7SK snRNA γ-methylphosphate capping enzyme
NER	Nucleotide excision DNA repair
NEXT	Nuclear exosome targeting complex
NELF	Negative elongation factor
NET-seq	Native elongating transcript sequencing
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
N-TEF	Negative transcription elongation factor
p300	Histone acetyltransferase p300
P-TEFb	Positive transcription elongation factor b
PAF1	RNA polymerase II associated factor 1
PCR	Polymerase chain reaction
PIC	Pre-initiation complex
PKA	cAMP-dependent protein kinase
PKCθ	Protein kinase C-theta
Pol II	RNA polymerase II
PP1α	Serine/threonine-protein phosphatase PP1-alpha
RBM7	RNA binding motif protein 7
RBP	RNA binding protein
RIP	RNA immunoprecipitation
RT-qPCR	Reverse transcription followed by quantitative PCR
SEC	Super elongation complex
Ser-2P	Phosphorylation at Serine 2 residues of the C/terminal domain of Pol II
Ser-5P	Phosphorylation at Serine 5 residues of the C/terminal domain of Pol II
s.e.m.	The standard error of the mean
SRSF2	Serine and arginine-rich splicing factor 2
Tet	Tetracycline hydrochloride
Tat	Trans-activator of transcription
Tax	Tax gene product
TC-NER	Transcription-coupled nucleotide excision DNA repair
TFIIH	Transcription factor II H
TES	Transcription end site
TP53	Tumour protein p53 gene
TSS	Transcription start site

uaRNA	Upstream antisense RNA
UV	Ultraviolet light
WDR43	WD repeat domain 43
XP	Xeroderma pigmentosum

6 INTRODUCTION

Gene expression is a fundamental feature of all living organisms. Transcription or synthesis of RNA from DNA is a crucial element of this process. RNA serves as an evolutionarily conserved intermediate between genetic information stored in DNA and proteins, which support functions of biological systems. Thus, gene transcription is often an initial step of any function of a biological system.

DNA-dependent RNA polymerases are molecular machines that synthesize coding and non-coding RNA by merging free nucleotides into a chain using DNA as a template. There are three major RNA polymerases in human cells. Pol I synthesizes a pre-ribosomal 45S RNA, which matures into 28S, 18S, and 5.8S rRNAs, which form a frame of the ribosome. Pol II synthesizes precursors of mRNAs and most snRNA and microRNAs. Pol III synthesizes transport RNAs, rRNA 5S and other small RNAs (Roeder, 2019). Pol II is the most studied RNA polymerase. Human Pol II consists of twelve protein subunits. The largest Pol II subunit, Rpb1, binds DNA and catalyzes RNA synthesis (Bernecky et al., 2016). Importantly, the carboxy-terminal domain (CTD) of Rpb1, which contains heptapeptide repeats of Tyr-Ser-Pro-Thr-Ser-Pro-Ser amino acid residues, is central for Pol II subunits assembly and regulation (Buratowski, 2003; Suh et al., 2016). Pol II transcribes protein-coding genes to produce mRNA molecules, as well as critical regulatory non-protein coding RNAs, like long non-coding RNAs (lncRNAs) and enhancer RNAs (eRNAs) (Mayer et al., 2015).

The transcription by Pol II has been studied extensively. General transcription factors (GTFs), associate with Pol II and guide transcription initiation, pausing, elongation, and termination (Figure 1) (Orphanides et al., 1996). In particular, a set of GTFs including key TFIID and TFIIF recognizes promoter region on DNA, remodels chromatin structure and forms a pre-initiation complex (PIC) which enables the Pol II to bind gene promoter and initiate transcription (Sainsbury et al., 2015). CDK7 of TFIIH phosphorylates CTD of Pol II landmarking transcription initiation (Nilson et al., 2015). Pol II transcribes about 20-100 nucleotides from transcription start sites (TSS) before its elongation is paused by the multiunit negative transcription elongation factors (N-TEFs), consisting of negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) (Core and Adelman, 2019). The release of the paused Pol II is stimulated by positive transcription elongation factor b (P-TEFb), which is composed of the catalytic CDK9 kinase and a regulatory CycT1 or CycT2 subunit (Core and Adelman, 2019; Peterlin and Price, 2006). Upon its recruitment to the target gene promoters or enhancers, P-TEFb phosphorylates serine 2 residues (Ser2-P) within the CTD

heptapeptide repeats of the largest Pol II subunit, RPB1, as well as the NELF-E of NELF and the SPT5 subunit of DSIF, leading to productive Pol II elongation (Bacon and D'Orso, 2018; Suh et al., 2016). Pol II elongates the RNA transcripts by moving through the body of genes. At the same time, the nascent RNA transcript is processed co-transcriptionally into the functional RNA through 5′-capping and splicing (Herzel et al., 2017). Once Pol II reaches the signal of the transcription termination on the DNA template at the end of the gene, transcription termination factors trigger termination of RNA synthesis, cleavage, and poly-adenylation of the RNA molecule and dissociation of Pol II from the gene (Richard and Manley, 2009).

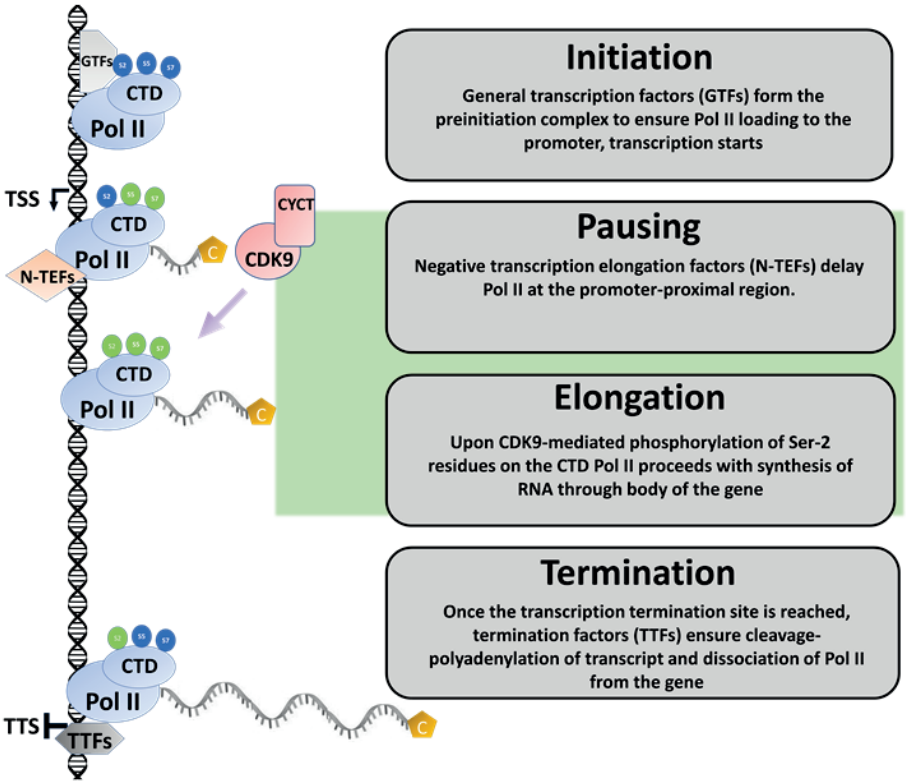

Figure 1. Focused overview of Pol II transcription. Phases of Pol II transcription are shown from top to bottom with the indicated transcription start site (TSS) and transcription end site (TTS) on the DNA strand. Grey boxes on the right contain a brief explanation of the indicated stages. Only selected TFs and steps of transcription are shown for clarity.

The gene transcription by Pol II is particularly responsible for the ability of cells to respond to internal and external signals, which modifies the functioning of cells in cellular and multicellular context. Dysregulation of Pol II transcription, which could be caused by genetic perturbations or pathogen influence, is a crucial element for many human diseases (Aso et al., 1996).

Importantly, the transition of Pol II from promoter-proximal pausing to productive elongation is a key regulatory step for many stimulus-responsive genes (Adelman and Lis, 2012; Henriques et al., 2013). Here, the P-TEFb activity towards Pol II CTD is critical (Price, 2000). Of note, a major fraction of P-TEFb resides within 7SK snRNP, in which coordinated actions of the scaffolding 7SK RNA and three RNA-binding proteins (RBPs) inhibit this kinase (Quaresma et al., 2016). While the 7SK γ-methylphosphate capping enzyme MePCE and La related protein 7 (LARP7) stabilize 7SK to form the core of 7SK snRNP (Barboric et al., 2009; He et al., 2008; Jeronimo et al., 2007), HEXIM1 subsequently interacts with 7SK of the core to recruit P-TEFb and directly inhibit its kinase activity (Michels et al., 2004; Yik et al., 2003).

In this thesis, we studied how cells modulate the gene expression program amidst the DNA damage response (DDR). DDR is a critical cellular process, that reorganizes functions of the cell to overcome stress-induced by damage of DNA (Jackson and Bartek, 2009). Proper DDR is essential for repair of damaged DNA through an appropriate mechanism (Jeggo et al., 2016) and for apoptotic elimination of those cells that carry too much of a mutational load to be repaired (Lanz et al., 2019). Insufficient DDR causes the accumulation of mutations that could lead to the malignant transformation of cells and cancer (Jeggo et al., 2016). Pol II transcription response is a critical step of DDR. It allows the rapid expression of important DDR factors, transcription-coupled DNA repair, and halting of the cell cycle progression (Giono et al., 2016). Particularly, transcription elongation is affected dramatically during DDR (Gregersen and Svejstrup, 2018). Surprisingly, given a central role of P-TEFb and 7SK snRNP in gene transcription elongation control (Li et al., 2005; Peterlin and Price, 2006), little is known about the functions and mechanisms of P-TEFb-dependent transcription during DDR. Considering that P-TEFb is crucial for the prompt expression of stimulus-induced genes we reasoned that it might feature prominently in activating Pol II transcription following DNA damage.

Given that cellular RBPs are emerging as important effectors in the DDR (Dutertre et al., 2014) and transcription (Quaresma et al., 2016; Skalska et al., 2017), we envisioned that a protein from this class could facilitate genotoxic-stress-induced Pol II transcription through 7SK snRNP and P-TEFb. In my dissertation, we studied the ubiquitously expressed RNA binding motif 7 (RBM7), the component of nuclear exosome targeting complex (NEXT), that binds short-lived RNA species for nucleolytic degradation (Lubas et al., 2011). Surprisingly, RBM7 also promotes the survival of cells following DNA damage generated by UV or its mimicking genotoxic and carcinogenic chemical 4-nitroquinoline 1-oxide (4-NQO) (Blasius et al., 2014; Ikenaga et al., 1977).

The thesis describes new factors that control the transition of Pol II from promoter-proximal pausing to elongation in response to DDR. Therefore, the literature review summarizes the current knowledge of regulating Pol II

transcription elongation by P-TEFb and 7SK snRNP. I have focused on the link between cellular DDR mechanisms and Pol II transcription, as well as the involvement of P-TEFb to the process. Besides this, I have provided background information on the NEXT complex and its unexpected link to the known DDR pathways. The summary of research results features our finding of a novel mechanism of DDR-mediated control of Pol II transcription via regulation of P-TEFb activity by 7SK snRNP and RBM7. Furthermore, we characterized the requirement of the P-TEFb-dependent gene transcription response during DDR for cell survival. Finally, I have discussed how the presented results extended our understanding of gene regulation following the DNA damage.

7 REVIEW OF THE LITERATURE

7.1. Pol II transcription elongation control by P-TEFb and 7SK snRNP

7.1.1. An overview of gene expression regulation at the stage of transition from promoter-proximal pausing to productive elongation

Since the rate of Pol II transcription is limited at all stages (Figure 1), the successful RNA synthesis initiation may not directly correspond to the productive gene expression. Instead of sliding along the bodies of genes in a linear process, Pol II elongation rather involves a complex chain of pausing, backtracking and resuming events. Initially, such irregular nature of transcription elongation consisting of pausing, stalling, and backtracking - the reversible sliding of RNA polymerase along the DNA, in addition to active elongation into the sense direction, was discovered in prokaryotes (Komissarova and Kashlev, 1997; Krummel and Chamberlin, 1992; Nudler, 2012). Therefore, resolving the structure of elongating Pol II in eukaryotic cells has confirmed that pausing and backtracking are essential processes during transcription (Bernecky et al., 2016; Cheung and Cramer, 2011). Particularly, a transcription elongation arrest occurs in the event of displacement of the 3′ end of RNA from the active site of Pol II while it slides back on DNA (Cheung and Cramer, 2011). To resume transcription the 3′ end of RNA is cleaved through the action of Rbp9 and transcription factor II S (TFIIS) resulting in a new 3′ end, which is aligned to DNA properly within an active site of Rpb1 (Izban and Luse, 1992; Lisica et al., 2016). The global occurrence of pausing was identified by sensitive genome-wide approaches involving nascent RNA sequencing and tracking of elongating Pol II (Churchman and Weissman, 2011; Core et al., 2008). Stalling of Pol II may occur because of local chromatin structure (Hodges et al., 2009), specific DNA sequences (Nechaev et al., 2010), reaching genetic landmark as exon-intron junction point (Laitem et al., 2015) or as a result of the interaction of transcribing machinery with DNA damage site (Brueckner et al., 2007), but the most important pausing events are associated with an early elongation (Core et al., 2008).

The pausing of Pol II at the promoter-proximal region is exceptionally significant and could limit the gene expression rates (Core and Adelman, 2019). The early studies of eukaryotic genes, such as *HSP70* from Drosophila (Rougvie and Lis, 1988), as well as human *MYC* (Strobl and Eick, 1992) and *FO*S (Kaneko et al., 1992; Krumm et al., 1992), later supported by genome-wide approaches (Core et al., 2008; Jonkers and Lis, 2015) emerged the field

of gene regulation at the stage of transition from promoter-proximal pausing to productive elongation. Interestingly, the promoters of promoter-proximally paused genes exhibit an open chromatin structure around the transcription start sites by continuous recruitment of Pol II complexes (Gilchrist et al., 2008; Gressel et al., 2019). Moreover, the transcription initiation factors, including TFIIH, Mediator complex, and other GTFs accompany Pol II at the promoter region of these genes (Allen and Taatjes, 2015; Core and Adelman, 2019; Takahashi et al., 2011). Meanwhile, Pol II produces only around 50 nucleotide-long transcripts before transcription slows down at the promoter-proximal region for pausing (Figure 2A) (Guo and Price, 2013; Zeitlinger et al., 2007). The establishment of the paused Pol II complex is an active process that requires two multi-subunit negative transcription elongation factors (N-TEFs): negative elongation factor (NELF) and DRB sensitivity-inducing factor (DSIF) (Fujita et al., 2009; Vos et al., 2018b; Wada et al., 1998; Yamaguchi et al., 1999).

Since about 70% of genes in metazoa show promoter-proximal pausing of Pol II to some extent (Churchman and Weissman, 2011; Core et al., 2012; Core et al., 2008) the pausing limits the rates of RNA synthesis for many essential genes and determines the global Pol II profile on the genes (Guo and Price, 2013; Mayer et al., 2015; Zhu et al., 1997). Moreover, the genes which exhibit higher rates of promoter-proximal pausing have shown to encode components of signal transduction pathways (Henriques et al., 2013; Kininis et al., 2009; Nechaev et al., 2010; Williams et al., 2015). This fact suggests that such a system of transcription control may be more efficient for fast and synchronous transcriptional responses. Indeed, the loading of polymerases ensures open chromatin structure and impacts nucleosome occupancy, providing an unobstructed path for active transcription (Adelman and Lis, 2012; Gressel et al., 2019; Henriques et al., 2013).

To proceed with the elongation of RNA, Pol II must be released from the pausing. P-TEFb is a primary activator of the promoter-proximal pause release in higher eukaryotes (Figure 2B). P-TEFb phosphorylates the Pol II CTD Serine-2 residues in its heptapeptide repeats as well as the NELF-E and Spt5 subunits of NELF and DSIF, respectively, which enables the release of Pol II from promoter-proximal pausing and thus the start of active transcription elongation (Peterlin and Price, 2006; Ping and Rana, 2001; Quaresma et al., 2016). The P-TEFb-mediated control of transcription elongation is critical, for example, for the heat shock genes (Eissenberg et al., 2007; Lis et al., 2000), HIV-1 transcriptional activation (Chao et al., 2000; Mancebo et al., 1997), as well as transactivation of oncogenes, like c-Myc (Gargano et al., 2007; Mitra et al., 2016).

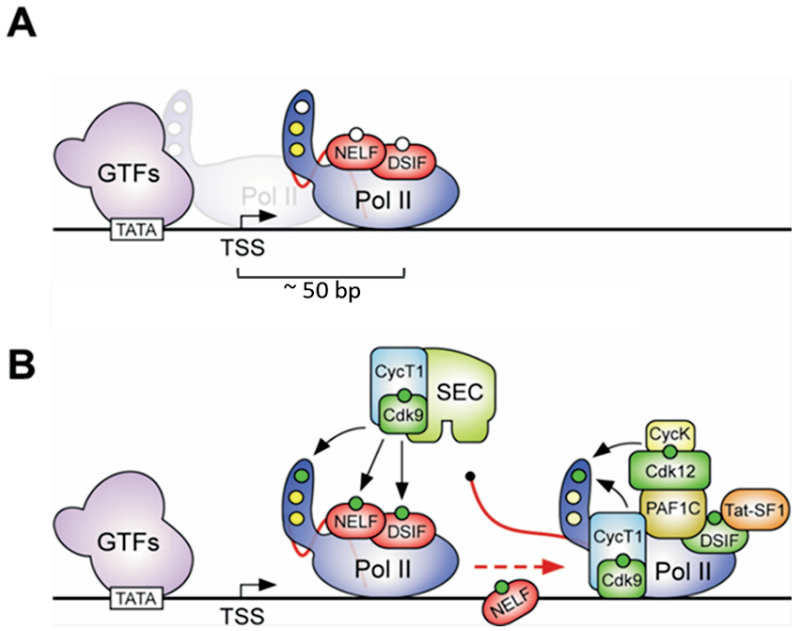

Figure 2. Pol II transcription control on the stage of transition from promoter-proximal pausing into productive elongation. A. Initiation of transcription and pausing. B. Release of Pol II from the pausing with the assistance of P-TEFb as part of the SEC. Additional CTD Ser-2P kinase CDK12 is recruited to phosphorylate Pol II at the bodies of genes by PAF1 complex. Adapted from Quaresma et al. 2016 with permission from the publisher (Quaresma et al., 2016).

The amount of Ser2-P Pol II signal increases towards the 3′ end of genes, suggesting that additional phosphorylation by CDK9 might take place along the elongation process (Laitem et al., 2015; Zaborowska et al., 2016). In addition, recent data indicate the involvement of the PAF1 complex in recruiting another Ser-2P CTD kinase – CDK12 (Yu et al., 2015). Of note, CDK12 and its cyclin partner cyclin K are critical for the transcription elongation of long DDR and core DNA replication genes (Figure 2B) (Blazek et al., 2011; Chirackal Manavalan et al., 2019; Ekumi et al., 2015). In summary, transcription elongation is a complex multistep process that involves several regulation points. The critical step for the expression of many genes is the release of Pol II from promoter-proximal pausing by P-TEFb, which phosphorylates the CTD of Pol II and N-TEFs.

7.1.2. P-TEFb as a critical regulator of promoter-proximal pause release

P-TEFb was initially identified in Drosophila as an essential factor for gene transcription after the initiation step (Marshall and Price, 1995). The high interest in P-TEFb was connected to the discovery of the requirement of its kinase activity for HIV-1 transcription (Mancebo et al., 1997; Wei et al., 1998). P-TEFb is a heterodimeric complex consisting of the catalytic subunit cyclin-dependent kinase 9 (CDK9) and the regulatory subunits cyclin T1 or T2 (CycT1/T2) (Marshall and Price, 1995; Peng et al., 1998a; Peng et al., 1998b; Wei et al., 1998). In addition to the two cyclin subunits, there also two CDK9 isoforms, the 42 kDa and 55 kDa, which are encoded by the same CDK9 gene transcribed from two alternative TSSs (Shore et al., 2005). Phosphorylation of CDK9 at Thr-186 is required for the maturation of the catalytic subunit of P-TEFb (Chen et al., 2004; Ramakrishnan et al., 2015). The activity of phosphatase PPM1A regulates the phosphorylation levels of CDK9 and the balance of active P-TEFb complexes in the cells (Wang et al., 2008).

The activity of P-TEFb towards the CTD of Pol II is critical for the promoter-proximal pause release (Laitem et al., 2015). CDK9 subunit of P-TEFb phosphorylates Ser-2 residues within heptapeptide repeats of Pol II CTD in the living cells to landmark the transition into productive elongation (Gressel et al., 2017; Mayer et al., 2015; Schüller et al., 2016). Interestingly, CDK9 phosphorylates Ser-5 residues of CTD predominantly in vitro, suggesting the complex nature of P-TEFb catalytic activities at the promoter-proximal paused Pol II (Czudnochowski et al., 2012). Critically P-TEFb also phosphorylates N-TEFs, NELF-E, and Spt5, allowing the displacement of these factors from the Pol II complex (Fujinaga et al., 2004; Wada et al., 1998; Yamaguchi et al., 1999). Of note, phosphorylation of the Spt5 subunit of DSIF is essential for polymerase associated factor 1 (PAF1) complex recruitment to the elongating Pol II, converting DSIF to transcription elongation promoting factor (Chen et al., 2009; Vos et al., 2018a; Wada et al., 2000). In addition, P-TEFb phosphorylates other substrates involved in gene transcription, such as transcription termination factor Xrn2 (Sanso et al., 2016).

The activity of P-TEFb is regulated by several mechanisms, including maturation of the P-TEFb components CDK9 and cyclins T by specific phosphorylation events, inhibiting the kinase activity of CDK9 within the inhibitory 7SK snRNP complex and recruitment of P-TEFb to target genes (Quaresma et al., 2016). To conclude, P-TEFb, as the main regulator of promoter-proximal pausing, is controlled at multiple levels to ensure temporally, spatially, and functionally correct gene transcription responses.

7.1.3. 7SK snRNP controls the activity of P-TEFb

To ensure tight transcription elongation control, up to 90% of all cellular P-TEFb in unchallenged cells resides in the repressed form within the 7SK snRNP complex. Initially, 7SK was discovered to be a critical component in the inhibition of the kinase activity of P-TEFb (Nguyen et al., 2001; Yang et al., 2001). 7SK is an abundant, 330-332 nucleotides long RNA, which is transcribed by RNA polymerase III mainly from the single RN7SK gene, despite a great number of pseudogenes encoding 7SK RNA exist in the human genome (Diribarne and Bensaude, 2009; Wassarman and Steitz, 1991).

The U-rich 3'-terminus of nascent 7SK is an attractive target for nucleolytic degradation (He et al., 2008). During its biogenesis, this part of 7SK forms a complex with LA protein, which probably stabilizes the RNA (Chambers et al., 1983). Upon binding to the 5'-terminus of 7SK, methyl-phosphate capping enzyme (MePCE, also known as BCDIN3) adds a methyl-phosphate cap and promotes an exchange of the LA protein to the La-related protein 7 (LARP7) on the 3'-terminus (Quaresma et al., 2016). The biogenesis stages, as well as the place of the proteins on the 7SK snRNA secondary structure, are indicated in Figure 3.

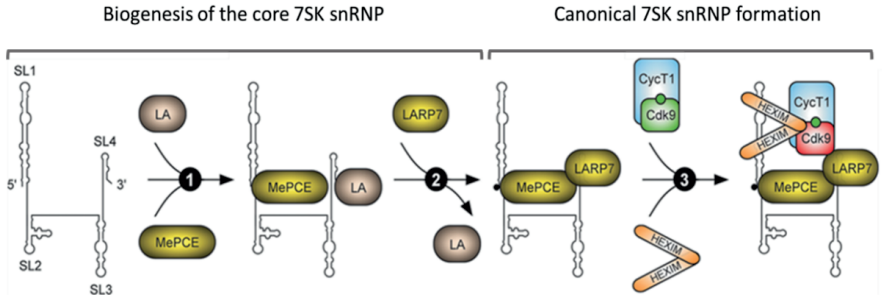

Figure 3. Biogenesis of 7SK snRNP. From left to right: 7SK snRNA is transcribed by Pol III and forms secondary structure consisting of four stem-loops (SL1-4); LA prevents 7SK nucleolytic degradation by binding its 3′-terminal U-rich sequence and MePCE promotes methyl-phosphate capping of the 5′ end of 7SK snRNA, respectively (Step 1). The capping provokes the replacement of LA with LARP7 protein at SL4 promoting the stability of 7SK snRNA. Moreover, LARP7 binds MePCE also directly (Step 2). HEXIM1/2 dimers bind the proximal and distal parts of 7SK, leading to the trapping of the active P-TEFb which is phosphorylated at Thr-186 (a green circle on CDK9) within the canonical 7SK snRNP (Step 3). Adapted from Quaresma et al. 2016 with permission from the publisher (Quaresma et al., 2016).

The mature core of the 7SK snRNP complex consists of 7SK snRNA and two RBMPs: LARP7 and MePCE, collectively promoting the stability of this snRNA (Barboric et al., 2009; He et al., 2008; Jeronimo et al., 2007). Depletion of these proteins with siRNA results in a decrease of cellular 7SK snRNA levels and disorganization of transcription elongation control (Barboric et al., 2009). When recruited into the complex, P-TEFb is directly repressed by another protein – hexamethylene bisacetamide-induced protein 1 (HEXIM1), which is

present within the vast majority of cellular 7SK snRNP complexes in a homodimeric form (Blazek et al., 2005; Yik et al., 2003). Interestingly, HEXIM1 could be replaced by its paralog HEXIM2 (Blazek et al., 2005; Michels et al., 2004; Yik et al., 2003). This HEXIM1-containing 7SK snRNP complex of P-TEFb represents its inactive fraction and provides possibilities for regulation (Bacon and D'Orso, 2018; Van Herreweghe et al., 2007). Also, alternative 7SK snRNP complexes exist. For example, in the conditions of induced P-TEFb and HEXIM1 release from the complex, hnRNPs bind the core 7SK snRNP consisting of 7SK, LARP7, and MePCE (Barrandon et al., 2007; Flynn et al., 2016; Krueger et al., 2008). Of note, before assembly into 7SK snRNP, CDK9 is phosphorylated at Thr-186, which is a hallmark of active cyclin-dependent kinases, suggesting that P-TEFb within 7SK snRNP is in a pre-activated form (Chen et al., 2004; Wang et al., 2008). In response to exogenous or endogenous signals, this active form of P-TEFb could leave the 7SK snRNP complex to promote the Pol II promoter-proximal pause release and rapid transcriptional induction (Core and Adelman, 2019; Quaresma et al., 2016).

The release of P-TEFb from 7SK snRNP triggers various Pol II transcriptional responses, which depend on the nature of the releasing stimulus . Accordingly, several molecular mechanisms have been shown to promote the release of P-TEFb from 7SK snRNP (Calo et al., 2015; Van Herreweghe et al., 2007). Historically, HIV-1 Tat protein was the first identified factor capable of releasing P-TEFb from 7SK snRNP to promote Pol II transcription elongation of HIV-1 transcripts (Barboric et al., 2007; Muniz et al., 2010; Sedore et al., 2007). Table 1 provides a summary of known molecular pathways promoting the release of active P-TEFb from the 7SK snRNP complex, as well as the direct P-TEFb release factors that have been found to date. Given that the nature of mechanisms involved in the releasing of P-TEFb from 7SK snRNP varies drastically in between factors identified by researchers, there is the possibility that many mechanistic details are missing in our understanding of this process (Quaresma et al., 2016).

Table 1. Summary of the molecular pathways and factors involved in the release of P-TEFb from 7SK snRNP.

Factor	Mechanism	Reference
Molecular signaling pathways favoring P-TEFb release from 7SK snRNP		
PKCθ	In response to the T-cell receptor signaling PKCθ prevents HEXIM1 from binding to 7SK snRNA by phosphorylation at Ser-158.	(Fujinaga et al., 2012)
ERK	ERK-dependent kinase phosphorylates HEXIM1 Tyr271 and Tyr274 to prevent CycT1/P-TEFb binding.	(Kim et al., 2011)
Akt	Activation of PI3K/Akt pathway induces phosphorylation of HEXIM1 at Ser-270 and Thr-278 by Akt to prevent CycT1/P-TEFb binding.	(Contreras et al., 2007)
PKA	cAMP-signaling induce phosphorylation of HEXIM1 at Ser-158 by PKA.	(Sun et al., 2019)
PP1α	PP1α dephosphorylates P-Thr186 in CDK9 after the remodeling of 7SK snRNP by PP2B to disable P-TEFb binding to the complex.	(Chen et al., 2008)
Calpain 2	Ca^{2+}-dependent calpain 2 cleaves MePCE to destabilize core 7SK snRNP.	(Elagib et al., 2013)
P-TEFb release factors		
HIV-1 Tat	Binds the distal part in SL1 of 7SK and CycT1 to dislodge HEXIM1; recruits PPM1G to dephosphorylate P-Thr186 Cdk9.	(Muniz et al., 2010)
HTLV-1 Tax	Binds CycT1 to dislodge HEXIM1.	(Cho et al., 2010)
NF-κB	NF-κB activation promotes the recruitment of PPM1G phosphatase for the dephosphorylation of P-Thr186 in CDK9.	(Barboric et al., 2001)
SRSF2	In complex with nascent RNA, SR-proteins bind SL3 of 7SK snRNA and trigger the P-TEFb release from 7SK snRNP.	(Ji et al., 2013)
DDX21	Through RNA helicase activity DDX21 changes the conformation of 7SK snRNA.	(Calo et al., 2015)

hnRNPs	Bind SL3 of 7SK to enable the subsequent P-TEFb release.	(Barrandon et al., 2007)
JMJD6	At super-enhancers JMJD6 demethylates the 7SK snRNA cap which destabilizes the 7SK snRNP.	(Liu et al., 2013)
p300	Acetylates CycT1 to dislodge HEXIM1.	(Fu et al., 2007)
WDR43	Binds 7SK snRNA and LARP7 at the promoters of transcriptionally active genes to dislodge P-TEFb from the complex with HEXIM1	(Bi et al., 2019)

7.1.4. Recruitment of P-TEFb to target genes

Even though for a fraction of genes P-TEFb is recruited to target genes as a component of 7SK snRNP (Bacon and D'Orso, 2018; Eilebrecht et al., 2014; Liu et al., 2013; McNamara et al., 2016), recruitment of the catalytically active P-TEFb to the target genes subsequently to its release from the 7SK snRNP pool is essential for its functions. Indeed, from the very initial studies showing that HIV-1 Tat protein efficiently recruits P-TEFb to the paused Pol II at the HIV-1 proviral DNA through interacting with the trans-activation response (TAR) RNA element of the nascent viral transcript, many other cellular factors have been shown to play an essential role in this recruitment process (Barboric et al., 2007; D'Orso et al., 2012). Such transcription co-factors as BET family acetyl-lysine recognizing chromatin adaptor protein (BRD4) capture free P-TEFb to the controlled active promoters (47-49). BRD4 contains the P-TEFb interacting domain with high structural similarity to HIV-1 Tat, which promotes P-TEFb capturing and two bromodomains with a high affinity to acetylated histones (Itzen et al., 2014; Yang et al., 2005). Moreover, pathway-specific classical transcription factors (TFs), for instance, p53, NF-kB, CIITA, and c-Myc, were shown to promote the recruitment of active P-TEFb to its target genes (Barboric et al., 2001; Felton-Edkins et al., 2003; Gargano et al., 2007; Kanazawa and Peterlin, 2001; Kanazawa et al., 2003). Since the P-TEFb requirement for transcription of many genes, it is likely that other, yet unknown transcription factors may populate this list.

Another layer of P-TEFb regulation relates to the participation of it as an integral component in various super elongation complexes (SECs) which regulate transcription elongation checkpoints for target genes (Jang et al., 2005; Yang et al., 2005; Yoshida et al., 2015). SEC typically consists of P-TEFb and various sets of Pol II transcription elongation factors such as 11-19 Lys-rich leukemia ELL family members (ELL1, ELL2, ELL3), the AFF4/FMR2

SEC-scaffolding family members AFF1 and AFF4, as well as ENL and AF9 proteins (Bacon and D'Orso, 2018; Lin et al., 2010; Scholz et al., 2015; Schulze-Gahmen et al., 2013b). Free P-TEFb from the nucleoplasm is recruited to the SECs through the interaction of the CycT1/T2 subunit with the cyclin binding domain of AFF4 (Schulze-Gahmen et al., 2013a). The efficiency of promoter-proximal release by P-TEFb is enhanced within the SEC because of the action of additional elongation-promoting factors of the ELL family (He et al., 2010). The number of multiunit SECs exists and promotes the Pol II transcription elongation locally at the target genes in a coordinated manner depending on a cell type and physiological condition (Bacon and D'Orso, 2018; Dahlberg et al., 2015).

7.1.5. P-TEFb-mediated transcription in cancer and CDK9 as a therapeutic target

The control of gene transcription is frequently perturbed in cancer. Cancer cells often depend on oncogene-driven transcription for the survival, proliferation, and metabolism (Bradner et al., 2017). Given that transcription rates of many genes depend on promoter-proximal pause release P-TEFb is involved extensively in cancer-driven transcription control (Franco et al., 2018). Indeed, several studies show that functional P-TEFb is required for the survival and proliferation of cancer cells (Morales and Giordano, 2016).

The link between P-TEFb and oncogene-mediated transcription was discovered initially by studies on the c-Myc oncogene (Kanazawa et al., 2003). P-TEFb is needed both for transcription of this factor and for the activation of c-Myc-controlled transcription, as this oncogene recruit P-TEFb to its target genes (Gargano et al., 2007). The studies of SEC complexes, which, besides P-TEFb, contain the transcription co-factors often mutated in various human leukemias, showed the dependency of these cancers on Pol II transcription elongation (Lin et al., 2010; Yokoyama et al., 2010). DNA translocations in MLL and ELL member genes of SEC result in the activation of hyperproliferative transcription programs, which are dependent on P-TEFb kinase activity (Benedikt et al., 2011; Shinobu et al., 1999). In addition to oncogene-driven transcription, P-TEFb activity is essential for the expression of pro-survival genes, which are critical for continuous cancer cell proliferation (Cao et al., 2017). For instance, BCL-2 family member MCL-1 (Haaland et al., 2005; Pietrzak and Puzianowska-Kuznicka, 2008) an established chemoresistance factor in many cancers, is highly dependent on P-TEFb (Cao et al., 2017; O' Reilly et al., 2018).

The field of targeting cancer-related transcription with inhibitors of transcription kinases has started from pan-CDK inhibitors, which target several CDKs because of their structural similarity (Baumli et al., 2008; Chao

and Price, 2001; Chen et al., 2016; Nilson et al., 2015). Nevertheless, clinical trials for the majority of CDK9 inhibitors were held at phase I (Morales and Giordano, 2016), and only flavopiridol and dinaciclib were tested in phase II trials for treatment of acute myeloid and chronic lymphocytic leukemia (Gojo et al., 2013; Karp et al., 2012; Lanasa et al., 2015). The major limitation of using these CDK9 inhibitors in the clinic identified in these studies was a high level of toxicity of such treatment. In recent years, however, highly specific CDK9 inhibitors, together with novel CDK-targeting chemical degradation approaches, have expanded the possibilities for therapeutic inhibition of P-TEFb in various cancer types (Cao et al., 2017; Czudor et al., 2018; Mitra et al., 2016; Morales and Giordano, 2016; Rahaman et al., 2018).

7.2. Cellular DNA damage response

DNA damage occurs frequently in our cells due to internal, such as the generation of reactive oxygen species or topoisomerases activity (Lindahl and Barnes, 2000), and external factors, such as certain chemicals capable of making covalent changes in DNA molecules or physical agents, like ultraviolet (UV) irradiation (Sinha and Häder, 2002). Cells react to DNA damage by activating molecular signaling cascades, ensuring cell survival and the integrity of the genome (Jackson and Bartek, 2009).

7.2.1. A general model of DDR

The model of cellular DDR includes DNA damage sensing, intracellular signal transduction, and execution of cellular response programs, that eventually result in DNA repair (Jackson and Bartek, 2009). Depending on the type of DNA damage, the elements of DDR may consist of various sets of cellular factors and molecular processes (Table 2).

Table 2. The summary of DDR mechanisms

Type of DNA damage	Sensing	Signal Transduction	Response	Reference
DNA bases mismatch, nucleotide modifications	MSH2, MSH3, MSH6, DNA glycosylases	MLH1, ABL1	Cellular response: P53, P73, CDKs Repair: EXO1, DNA polymerases δ and ε, PCNA, RFC, RPA, ligase I, AP endonucleases	(Kim and Wilson, 2012; Liu et al., 2017)
Bulky DNA adducts, UV-photoproducts	Pol II, XPE/C	p38 MAPK, ATR, ATM, MK2	Cellular response: P53, CDKs Repair: TFIIH, CSA, CSB, RPA, ligase I	(Schärer, 2013)
Single-strand DNA breaks	DNA glycosylases, PARP1, PARP2	ATR, ATM, CHK1, CHK2.	Cellular response: P53, CDKs Repair: XRCC1, RPA, ligase I, DNA polymerases	(Iyama and Wilson, 2013)
Double-strand DNA breaks	Ku70/80, DNA-PKcs, MRN complex	ATM, ATR, CHK1, CHK2, PARP1	Cellular response: P53, CDKs Repair: XRCC4, XLF, RAD51, BRCA1 and many more.	(Scully et al., 2019)

Here, I present an overview of DDR to the bulky DNA lesions as an example (Figure 4). Sensing of DNA damage is a crucial stage of DDR. Depending on the type of DNA damage cells evolved several systems, which sense DNA double-strand or single-strand breaks as well as bulky DNA lesions, for instance, 6-4 photoproducts (McGowan and Russell, 2004). In the latter case, the sensing of damaged DNA can be achieved by elongating Pol II, which stalls at the damaged sites (Gregersen and Svejstrup, 2018; Wang et al., 2018). The stalled transcription machinery recruits the factors for transcription-coupled DNA repair, such as Cockayne syndrome CSA and CSB proteins (also known as ERCC-8 and ERCC-6, respectively), as well as transcription factor TFIIH complex (Compe and Egly, 2012). DNA modifications within the non-transcribed genomic location are targeted by global nuclear excision repair. Several sensor proteins, for instance, Xeroderma Pigmentosum proteins XPE and XPC, identify such DNA lesions (Le May et al., 2010; Sugitani et al., 2016).

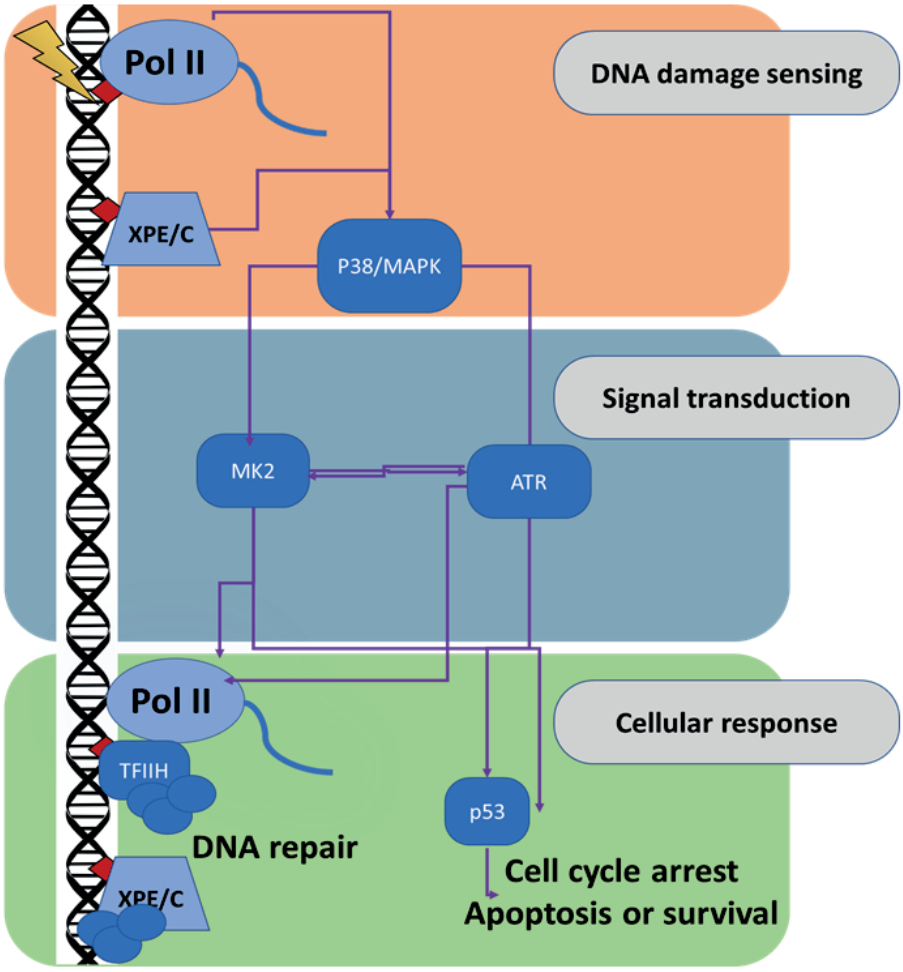

Figure 4. Simplified view on cellular DDR to bulky DNA lesions. Logical phases of DDR are depicted with colored areas and named accordantly. Examples of Pol II and XPA/XPC shown for DNA damage sensing. Signal transduction and some important intermediates (MAPK, ATR) leading to cellular response represent complex cascades of networks involved. Cellular responses are in large dependent on p53 activity as well as DNA repair pathways.

The detection of DNA damage activates cascades of DDR signal transduction networks led by protein kinases (Lanz et al., 2019) like Ataxia Telangiectasia Mutated serine/threonine (ATM) kinase, Ataxia Telangiectasia And Rad3-Related Protein serine/threonine (ATR) kinase or p38 Mitogen-Activated (p38 MAPK) kinase (Manke et al., 2005; Reinhardt et al., 2007). These kinases phosphorylate the target proteins leading to activation of cellular response directed by p53, cell cycle checkpoints (Bulavin et al., 2001) and specific DNA repair pathways accordingly to the type of DNA damage (Bruno et al., 2006). For instance, signal transduction in the case of bulky DNA

adducts involves the p38 MAPK pathway, as well as ATR, activate MK2 and checkpoint kinases CHK1/2 leading the cellular response to DNA damage (Borisova et al., 2018; Kim et al., 2002).

DDR induces several cellular stress responses, including cell cycle arrest, inhibition of RNA and protein synthesis, metabolic changes, and programmable cell death (Schuler and Green, 2005). This process, controlled by stress-induced transcription factors such as p53, ensures the elimination of the cells, which failed to restore genetic integrity and provides the cellular environment for DNA repair processes (Dregoesc et al., 2007; Jackson and Bartek, 2009; Schuler and Green, 2005). Therefore, DNA repair is an essential stage of cellular DDR aimed to eliminate the mutations and restore the genome integrity of exposed cells. For instance, bulky DNA lesions induced by UV irradiation are removed through nucleotide excision repair (NER) (Jackson et al., 1994; Schärer, 2013). There are two types of NER that differ based on the DNA lesion detection mechanism: transcription-coupled NER (TC-NER), where stalled Pol II in the complex with CSB protein serve as a DNA damage sensor and global NER, where this function is performed by XPE and XPC proteins (Le May et al., 2010). Subsequent recruitment of TFIIH and RPA followed by the removal of the short fragment of the damaged strand of DNA and DNA synthesis based on the undamaged DNA strand are common steps for both types of NER (Compe and Egly, 2012; Vermeulen and Fousteri, 2013).

7.2.2. DDR and Pol II transcription

Pol II transcription involved in DDR through several different mechanisms. Firstly, Pol II transcription machinery may directly interact with the sites of DNA damage. Depending on the type of lesion the consequences of such interaction ranging from the bypass of DNA lesion to Pol II stalling and backtracking (Shanbhag et al., 2010). One of the best-studied examples of direct interaction of Pol II transcription with DNA damage is a transcription stalling at the sites of DNA damage caused by UV light (Gregersen and Svejstrup, 2018). The UV light can induce the formation of pyrimidine dimers of nucleotides and other photoproducts in DNA molecules (Jackson et al., 1994; McKay et al., 1997). Likewise, the treatment of cells with such agents as 4- nitroquinoline oxide (4-NQO) induces a similar type of DNA damage. Specifically, 4-NQO is metabolized in cells into 4-hydroxyaminoquinolone 1-oxide, which forms DNA-adducts with purine residues (Ikenaga et al., 1977). Transcribing Pol II machinery which physically encounters such sites stalls at them and provides a platform for the transcription-coupled DNA repair pathway (Brueckner et al., 2007; Chiou et al., 2018; Donahue et al., 1994; Rockx et al., 2000; Vermeulen and Fousteri, 2013). TC-NER was shown to be dependent on Pol II is exclusively active in transcribed strands and mostly

takes place at the beginning of the genes (Hu et al., 2015). Interestingly, Pol II, which is essential for sensing of UV-induced DNA damage, needs to be removed from the DNA lesion sites for efficient repair (Gregersen and Svejstrup, 2018). Therefore, stalled elongating Pol II complexes could be removed through the activity of CSB and TFIIH for subsequent ubiquitylation and degradation (McKay et al., 2001b; Wilson et al., 2013).

Alternatively, Pol II serves also as an effector of DDR pathways, which modulate the transcription initiation and elongation, and consequently RNA synthesis output (Gregersen and Svejstrup, 2018). In the case of UV-like DNA damage, overall RNA synthesis, regardless of the physical interaction of Pol II with DNA lesions, is down-regulated (Boeing et al., 2016; Rockx et al., 2000) that is explained by a need to shut down production of aberrant (based on mutated DNA sequence) RNA transcripts (Pirngruber and Johnsen, 2010). Namely, the processivity of Pol II elongation in response to bulky DNA lesions progressively diminishes towards the TTS (Andrade-Lima et al., 2015; Giono et al., 2016; Heine et al., 2008; McKay et al., 2004; Wilson et al., 2013). Therefore, co-transcriptional RNA splicing is perturbed because of slow elongation at long genes leading to the increased usage of weak splicing sites (Muñoz et al., 2009). In addition, transcription blocking DNA damage induces spliceosome displacement from the chromatin leading to activation of ATM (Tresini et al., 2015).

In contrast to overall RNA synthesis inhibition described above, upon genotoxic stress Pol II actively transcribes genes encoding various DDR factors, which are as usual represented by exclusively short transcriptional units (Allen et al., 2014; Huang et al., 1998; Shams et al., 2013). Likewise, the genome-wide metagene data also indicates an increase of Pol II transcription following the UV light exposure within approximately 10 kbp from TSSs, probably representing the permissive window of DDR-induced transcription (Andrade-Lima et al., 2015; Lavigne MD, 2017; Williamson et al., 2017). The normal Pol II transcription state is restored at the final stage of DDR after the damage of DNA is repaired (Gregersen and Svejstrup, 2018; Oksenych et al., 2013; Rockx et al., 2000). Taken together, these observations suggest that DDR involves both activation and repression of Pol II transcription. Pol II transcription is integrated into various molecular networks of the DDR including both DNA damage sensing and the cellular response to DNA damage.

7.2.3. The role of p53 in governing DDR

p53 is a key TF of cellular response to various cell stress conditions (Kern et al., 1991). TP53, which encodes the p53 protein, is one of the critical tumor suppressor genes in humans with a high mutation rate in human cancers

(Kandoth et al., 2013). p53 is within the heart of the molecular network regulating cell fate upon stress conditions, like DNA damage (Kastenhuber and Lowe, 2017). Particularly, p53 binds to promoters of target genes to regulate gene expression response to the cell stress signals (Allen et al., 2014; Andrysik et al., 2017; Quaas et al., 2012). The protein levels of p53 are highly regulated in cells through degradation by ubiquitin ligase MDM2. Stress condition triggers phosphorylation of p53 at several sites within its transactivation domain, via upstream signal transduction kinases like ATM/ATR, or p38 MAPK, inhibiting its interaction with MDM2, which leads to p53 accumulation and prompt execution of its transcriptional program (MacLaine and Hupp, 2011; Zeng et al., 2000). For example, p53 directly activates transcription of CDKN1A gene encoding p21(WAF1) inhibitor of cyclin-dependent kinases such as CDK1/2/4/6, which induces cell cycle arrest (Fischer et al., 2014; Lohr et al., 2003; Sullivan et al., 2018). p53-driven transcription is also responsible for inducing apoptosis as the expression of pro-apoptotic proteins, like BAX or BAD, depends on activated p53 (Fortuno et al., 1999; Yamaguchi et al., 2004).

p53 is a potent tumor suppressor since its capacity to promote cellular and genetic homeostasis via controlling the cell cycle and apoptosis (Kastenhuber and Lowe, 2017; Schuler and Green, 2005). Upon activation, p53 is capable to initiate cell cycle arrest to disable proliferation and initiate programmable cell death in genetically and metabolically corrupted cancer cells (Hientz et al., 2017; Racay et al., 2008). Therefore, TP53 mutations have a wide-spread occurrence in almost every type of cancer with the highest rates of up to 50% in ovarian, esophageal, colorectal, lung, and larynx cancers and lower rates of around 5% for primary leukemia, testicular cancer, and cervical cancer (Kastenhuber and Lowe, 2017; Olivier et al., 2010). That is why various ways to activate the p53 pathway or restore mutant p53 activity are important directions in the cancer field (Abarzua et al., 1996; Hientz et al., 2017). For example, 5-fluorouracil (5-FU) is used for chemotherapy as it dysregulates nucleic acid synthesis and activates p53 indicating that the efficiency of therapy will rely on the TP53 status (Chuang et al., 2012; Heidelberger et al., 1957). Alternatively, the p53-MDM2 interaction could be attenuated with small molecules such as Nutlin-3a, inducing p53 protein accumulation and consequently, p53 pathway activation without any actual cell stress condition or accumulation of DNA damage (Cheok and Lane, 2017; Kojima et al., 2006; Sullivan et al., 2012).

7.3. Nuclear exosome targeting complex and RBM7

7.3.1. Overview of functions of RBM7 as a component of nuclear exosome targeting complex

The nuclear exosome is a multi-protein complex that degrades various types of RNA molecules inside the nucleus using 3'-exo/endoribonucleolytic activity (Kilchert et al., 2016; Mitchell et al., 1997). Nuclear exosome has no specificity and depends on different types of additional adapter complexes to recognize the target RNA molecules (Kilchert et al., 2016; Lubas et al., 2011). These adapter complexes usually contain RNA helicase hMTR4, which unwinds target RNAs to make it accessible for exosomal degradation and RBPs, which bind target RNAs (Schmid and Jensen, 2018).

The nuclear exosome targeting complex (NEXT) is a trimeric complex consisting of RNA-binding motif protein (RBM7), zinc-knuckle protein-adaptor ZCCHC8, and DExH/D box RNA-helicase hMTR4 (Lubas et al., 2011). NEXT complex was first identified as essential for RNA exosome-mediated degradation of short-lived RNA transcripts inside the nucleus. These RNAs are upstream antisense RNAs (uaRNAs) – side products of the transcription of the protein-coding gene from divergent promoters, as well as eRNAs originating from the active enhancers (Flynn et al., 2011; Preker et al., 2008). RBM7 is an essential RBP of NEXT complex, which with the assistance of its conserved RNA-binding RNP1 domain targets RNA-helicase hMTR4 to various types of short-live RNA (Falk et al., 2016; Hrossova et al., 2015). In addition, several studies revealed the physical and functional link on NEXT complex to the Cap-binding complex, which is associated with 5'-cap of nascent RNA suggesting that NEXT may be involved in the elimination of aberrant Pol II transcripts (Andersen et al., 2013; Lubas et al., 2015).

7.3.2. RBM7 in cellular DDR

Despite the role of RBM7 of the NEXT complex in the control of RNA degradation by the nuclear exosome, RBM7 is also involved in DDR. RBM7 was identified in the DNA damage-induced interaction screen of 14-3-3 protein, which functions for bringing different components of DDR signaling together (Blasius et al., 2014; Reinhardt and Yaffe, 2013). Such DNA damaging agents as UV or 4-NQO induce phosphorylation of RBM7 on Ser-136 and Ser-204 residues by MK2 kinase in response to activation of the p38 MAPK pathway (Blasius et al., 2014; Reinhardt et al., 2007). Upon its phosphorylation, RBM7 is losing interaction with the uaRNAs, leading to the

accumulation of these transcripts in the cells because of the impaired function of NEXT (Tiedje et al., 2015). Meanwhile, the genetic knockdown of RBM7 sensitizes the cells to genotoxic agents, including UV light and 4-NQO (Blasius et al., 2014). Therefore, it is little known about the molecular mechanism of the RBM7 requirement for cell survival under genotoxic attack. Of note, RBPs are often identified in DDR screens, for example, as substrates of ATM and ATR kinases (Matsuoka et al., 2007) and may be responsible for various functions during DDR (Dutertre et al., 2014; Dutertre and Vagner, 2017). RBPs were shown to sequester directly an interaction of DNA repair factors at the sites of DNA damage (Polo et al., 2012; Rulten et al., 2014) as well as trigger DDR through RNA-mediated functions (Francia et al., 2012; Khanduja et al., 2016). Likewise, since DNA damage triggers NEXT displacement from uaRNAs and the accumulation of these RNA species, RBM7 could be involved in DDR through regulation of the ncRNA metabolism.

7.4. Gaps of knowledge in the Pol II transcriptional response to DNA damage

Given the complex nature of the cellular DDR, the role of Pol II transcription in this process was studied from various angles. Here, the aspects of the Pol II transcription shutdown in the case of DNA adducts lesions are highly described, whereas the process of transcription activation is poorly understood (Gregersen and Svejstrup, 2018; Wilson et al., 2013). Interestingly, UV light, while inhibiting the global RNA synthesis rates (Rockx et al., 2000), induces also hyperphosphorylation of the CTD of Pol II and the activation of Pol II transcription within 10 kbp of TSS (Andrade-Lima et al., 2015; Williamson et al., 2017) suggesting that CTD kinases are involved in this process. Namely, P-TEFb was placed in the center of the UV-induced DDR network by a recent multi-omics study based on an unbiased analysis of the number of proteomic and functional genomic screens (Boeing et al., 2016). However, despite the number of studies on P-TEFb regulation upon DNA damage (Morawska, 2012; Napolitano et al., 2010), the function of P-TEFb in DDR is not yet defined. Of note, the release of P-TEFb from the inhibitory 7SK snRNP complex is associated with phosphorylation changes of the CTD of Pol II was described, in addition to UV, for several other cell stress conditions (Amente et al., 2009; Napolitano et al., 2010; Napolitano et al., 2013). It is not clear if the global P-TEFb activation upon cell stress is related only to activation of a minor subset of DDR genes, involved in TC-NER via interaction with DNA-repair factor CSB (Boeing et al., 2016; Proietti-De-Santis et al., 2006), or aimed for gene transcription restoration after DNA is repaired (Boeing et al., 2016; McKay et al., 2001b). One of the possible explanations of

such global reactivation of P-TEFb in different stress conditions is the involvement of this kinase into p53 functions. Critically, p53-mediated transcription relies on P-TEFb activity towards Pol II (Albert et al., 2016; Gomes et al., 2006; Napolitano et al., 2007; Radhakrishnan et al., 2008) and is vital for cell survival upon UV irradiation (McKay et al., 2001a; McKay and Ljungman, 1999). Inhibition of Pol II transcription, in turn, induces p53 activation (Derheimer et al., 2007; Ljungman et al., 1999; Shandilya et al., 2012), which may be a part of the same feedback loop involving P-TEFb as an activator of transcription. In summary, P-TEFb regulation of transcription represents an essential connection point in cell responses to DNA damage and understanding the mechanism of its action is the urgent direction of research within the field (Bacon and D'Orso, 2018; Bres et al., 2008).

8 AIMS OF THE STUDY

1. To dissect the mechanism of RBM7-mediated cell survival during DDR.

   Research objectives, related to this aim:
   - To dissect the set of RNAs, which specifically bind to RBM7 in response to DNA damage using iCLIP followed by sequencing.
   - To reveal novel protein partners of RBM7, related to its functions in DDR.
   - To validate the proposed mechanism using genetic and pharmacological approaches.

2. To uncover the functional significance of P-TEFb-dependent gene transcription response during DDR.

   Research objectives, related to this aim:
   - To assess the fast Pol II transcription response to DNA damage induced by 4-NQO using 4sU-sequencing followed by differential expression analysis.
   - To address the role of P-TEFb activity and RBM7 in DDR-induced transcription using pharmacological and genetic approaches.
   - To determine if P-TEFb-mediated gene transcription induced by DNA damage is essential for cell survival upon genotoxic insult.
   - To explore the possibilities of inducing the dependency of cancer cells on the P-TEFb-driven transcription through nongenotoxic activation of DDR.

9 MATERIALS AND METHODS

Detailed explanations of the experimental procedures, reagents, and approaches used in the current study are described in Article I. Here, I presented a summarizing overview of the materials and methods.

9.1. Materials

9.1.1. Table 3. Reagents used

Method	Reagent	Source
Chemicals and treatments	4-NQO	Sigma/Merck
Chemicals and treatments	Flavopiridol	Sigma/Merck
Chemicals and treatments	Tet	Sigma/Merck
Chemicals and treatments	B203580	Selleckchem
Chemicals and treatments	Doxycycline	Sigma/Merck
Chemicals and treatments	NVP-2	N. Gray lab (Dana-Farber Cancer Institute)
Chemicals and treatments	Nutlin-3a	Selleckchem
Chemicals and treatments	5-FU	Selleckchem
Chemicals and treatments	i-CDK9	Q. Zhou lab (UB Berkeley)
coIP, RNA IP, ChIP, WB	EDTA-free Protease Inhibitor Cocktail	Sigma/Merck
coIP, RNA IP, ChIP, WB	SUPERase In RNase Inhibitor	Thermo Fisher Scientific
coIP, RNA IP, ChIP	Protein G Dynabeads	Thermo Fisher Scientific
coIP, RNA IP, ChIP	Proteinase K	Thermo Fisher Scientific
CLIP-seq	Radiolabelled ATP, [α-32P]	Perkin Elmer
CLIP-seq	RNAse I	Thermo Fisher Scientific
CLIP-seq	CircLigase™ ssDNA Ligase	Epicentre
CLIP-seq, ChIP	Phenol:Chloroform:Isoamyl Alcohol 25:24:1	Sigma/Merck
4-sUseq	4-SU	Carbosynth
Molecular cloning	Phusion High-Fidelity DNA polymerase	NEB
Molecular cloning	Rapid DNA Ligation Kit	Thermo Fisher Scientific
Molecular cloning	Q5®Site-Directed Mutagenesis Kit	NEB
Molecular cloning	pcDNA5 FRT-TO-3XFLAG plasmid	J. Ule lab (The Francis Crick)
RNA extraction	Turbo DNA-Free kit	Thermo Fisher Scientific
RNA extraction	TRI reagent	Sigma/Merck
RT/qPCR, CLIPseq	SuperScript III reverse transcriptase	Thermo Fisher Scientific
RT/qPCR	Random hexamers	Thermo Fisher Scientific

RT/qPCR	MMLV reverse transcriptase	Thermo Fisher Scientific
RT/qPCR	FastStart Universal SYBR Green QPCR Master	Sigma/Merck
4sU-seq	EZ-Link Biotin-HPDP	Thermo Fisher Scientific
siRNA transfection	Lipofectamine RNAiMAX	Thermo Fisher Scientific
Protein purification	3XFLAG peptide	ApexBio Technology
RNA extraction	TRIzol LS	Thermo Fisher Scientific
4sU-seq	µMACS Streptavidin Kit	Miltenyi
CoIP	anti-FLAG M2 affinity gel	Sigma/Merck
Cell survival/cytotoxicity assays	CellTox Green Cytotoxicity Assay	Promega
Cell survival/cytotoxicity assays	RealTime-Glo Annexin V Apoptosis Assay	Promega
Cell survival/cytotoxicity assays	CellTiterGlo	Promega
Cell culture	Dulbecco's Modified Eagle Medium	Sigma/Merck
Cell culture	McCoy 5A Medium	Sigma/Merck
Cell culture	RPMI-1640 Medium	Sigma/Merck
Cell culture	Fetal Bovine Serum	Sigma/Merck
Cell culture	Penicillin-Streptomycin	Sigma/Merck

9.1.2. Table 4. Plasmid vectors.

Vector	Source
pcDNA5/FRT/TO/3XFLAG	Laboratory of J. Ule (The Francis Crick Institute)
pcDNA5/FRT/TO/3XFLAG-RBM7	Laboratory of J. Ule (The Francis Crick Institute)
pcDNA5/FRT/TO/3XFLAG-RBM7/mRNP1	Article I
pcDNA5/FRT/TO/3XFLAG-CDK9	Article I
pcDNA5/FRT/TO/3XFLAG-HEXIM1	Article I
pcDNA5/FRT/TO/3XFLAG-LARP7	Article I
pEF.YN/CTD	Laboratory of B.M. Peterlin (UCSF) (Fujinaga et al., 2015)
pEF-YC/P-TEFb	Laboratory of B.M. Peterlin (UCSF) (Fujinaga et al., 2015)
pET28a/MBP	Laboratory of M. Geyer (University of Bonn)
pET28a/MBP/ RBM7	Article I
pET28a/MBP/ RBM7/mRNP1	Article I
pGEX4T1/HEXIM1	Laboratory of M. Geyer (University of Bonn)
pGEX4T1/MePCE (aa 400-689)	Laboratory of M. Geyer (University of Bonn)
pGEX4T1/LARP7	Laboratory of M. Geyer (University of Bonn)
pGEX4T1/CycT1 (aa 1-272)	Laboratory of M. Geyer (University of Bonn)
pACEBac1/CDK9	Laboratory of M. Geyer (University of Bonn)

9.1.3. Table 5. Cell lines.

Cell line	Source
HEK 293 Flp-In T-Rex	Thermo Fisher Scientific
HEK 293 Flp-In T-Rex 3XFLAG	Article I
HEK 293 Flp-In T-Rex F-RBM7	Laboratory of J. Ule (The Francis Crick Institute)
HEK 293 Flp-In T-Rex F-RBM7 mRNP1	Article I
HEK 293 Flp-In T-Rex F-LARP7	Article I
HEK 293 Flp-In T-Rex F-CDK9	Article I
HEK 293 Flp-In T-Rex F-HEXIM1	Article I
HEK 293 Flp-In T-Rex F-MTR4	Hiraishi et al.
HeLa Flp-In	Laboratory of E. Bertrand (University of Montpellier)
HeLa Flp-In F-RBM7	Article I
HeLa Flp-In F-RBM7 mRNP1	Article I
HeLa	ATCC
RPE-1	ATCC
HFF-1	ATCC
HCT116 $TP53^{+/+}$ and HCT116 $TP53^{-/-}$	Laboratory of J.M. Espinosa (University of Colorado)

9.1.4. Table 6. Antibodies.

Antibody	Supplier
Mouse monoclonal anti-FLAG M2	Sigma
Rabbit monoclonal anti-Pol II RPB1	Abcam
Rabbit polyclonal anti-Pol II CTD repeat YSPTSPS [P-Ser2]	Abcam
Goat polyclonal anti-HEXIM1	Everest Biotech
Rabbit polyclonal anti-RBM7	Proteintech
Rabbit polyclonal anti-CDK9	Santa Cruz Biotechnology
Rabbit monoclonal anti-CDK9	Cell Signaling
Rabbit polyclonal anti-LARP7	Zhou Laboratory (UC Berkeley)
Goat polyclonal anti-MePCE	Santa Cruz Biotechnology
Mouse monoclonal anti-hnRNP A1	Abnova
Mouse monoclonal anti-g-H2AX (phosphor-S140)	Abcam
Rabbit polyclonal anti-MTR4	Abcam
Mouse monoclonal anti-EXOSC3	Santa Cruz Biotechnology
Rabbit polyclonal anti-Cyclin T1	Santa Cruz Biotechnology
Rabbit polyclonal anti-Cleaved PARP	Cell Signaling
Rabbit polyclonal anti-Cleaved Caspase-3	Cell Signaling
Mouse monoclonal anti-GAPDH	Santa Cruz Biotechnology
Rabbit monoclonal anti-phospho-p38 MAPK	Cell Signaling
Normal mouse IgG	Santa Cruz Biotechnology
Normal rabbit IgG	Santa Cruz Biotechnology
Normal goat IgG	Santa Cruz Biotechnology

9.2. Molecular cloning and mutagenesis

To generate plasmid vectors encoding 3XFLAG-tagged proteins the cDNAs were amplified using Phusion High-Fidelity DNA polymerase (NEB) with appropriate primers followed by molecular cloning using Rapid DNA Ligation Kit (Thermo Fisher Scientific) into the pcDNA5/FRT/TO-3XFLAG vector carrying triple FLAG-tag inside multiple cloning site. For the generation of point mutations inside the genetic constructs Q5® Site-Directed Mutagenesis Kit (NEB) was used where applicable. To construct plasmid vectors carrying the MBP-tagged RBM7 and mRNP1 RBM7 the respective cDNAs were inserted into a modified pET28a vector containing the MBP sequence on N-terminus followed by a TEV protease cleavage site. For the generation of baculoviral transfer vector encoding His-tagged CDK9, cDNA of CDK9 was amplified and inserted into pACEBac1 together with the OctaHis-tag encoding sequence. The cDNAs of full-length HEXIM1 and LARP7, as well as MePCE (aa 400-689) and CycT1 (aa 1-272) domains, were inserted into a pGEX4T1 vector containing TEV protease cleavage site between the GST tag and the protein. Table 4 lists plasmids and primers used to generate the plasmids.

9.3. Cell culture

Table 5 lists the cell lines used in the study and their origins. Human HeLa, human foreskin fibroblasts (HFF-1), and HEK 293 cells were grown in DMEM medium supplied with 10% FBS and penicillin-streptomycin mix (Pen-Strep). Human colon cancer HCT116 cells were grown in McCoy5A medium supplied with 10% FBS and Pen-Strep. Human retina pigment epithelium (RPE-1) cells were grown in RPMI medium supplied with 10% FBS and Pen-Strep.

To generate HEK 293 and HeLa Flp-In cell lines carrying genetic construct of interest, I used Zeocin (Invivogen), Blasticidin (Invivogen) and Hygromycin (Invivogen) according to the manufacturer's protocol. To induce expression of the FLAG-tagged protein in HEK293 Flp-In T-Rex cells I used tetracycline in the concentration of 1 μg/ml for 24 h.

9.4. Chemical compounds

The chemicals used during the study are listed in Table 3. Tetracycline hydrochloride (Sigma), which was used to induce expression of the tagged proteins in Flp-In cell lines. It was diluted using water as a solvent to a final concentration of 1 mg/ml and stored at -20°C. 4-thiouridine (4sU) from Carbosynth was used to label nascent RNA for 4sU-seq experiments. It was diluted in DMSO to a final concentration of 1 mM and stored at -20°C. Flavopiridol (Sigma) was used to inhibit CDK9 in the Article I. NVP-2 and SNS032-THAL CDK9 inhibitors diluted in DMSO were received from N. Gray's Lab, Dana Farber Institute (Boston, USA), i-CDK9 was a gift from Q. Zhou's Laboratory (UCSF in Berkley, USA). All these inhibitors were first diluted in DMSO to the concentration of 10mM and stored at -20°C. DDR-inducing agents: 4-NQO (Sigma), Nutlin-3a (Selleckchem), 5-fluorouracil (5-FU) (Selleckchem) were diluted in DMSO and used in concentrations indicated in figure legends. Triptolide (Selleckchem) was used to inhibit the XBP subunit of TFIIH and SB203580 (Selleckchem) inhibited p38 MAPK kinase. Both drugs were diluted in DMSO and stored in at -20°C.

9.5. UV irradiation

For UV crosslinking of protein-RNA complexes, Stratalinker 2400 equipped with a 254 nm wavelength lamp was used. The cells were crosslinked on the cell culture dishes in ice-cold PBS with a dose of 150 mJ/cm^2 of UV light. UV irradiation of the cells was performed in Crosslinker CL-1000 using a 254 nm wavelength lamp with doses of 10-40 mJ/cm^2 for DDR induction. The cell culture medium was replaced before UV treatment and added back immediately upon irradiation.

9.6. RNA interference, siRNAs and antisense-oligonucleotide transfection experiments

For RT-qPCR, co-IP, cytotoxicity, viability, and apoptosis assays, cells were transfected with siRNA for 48 h. 50 pmol siRNA was used per well of 6-well plate. 150 or 450 pmol siRNA was used to transfect 10 or 15 cm plates, respectively. 100 pmol phosphorothioate antisense DNA oligonucleotide against 7SK snRNA (as7SK) for 48 h was used to deplete 7SK snRNA.

Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) was used for siRNA transfections following the manufacturer's manuals. Sequences of siRNA and as7SK oligonucleotide are listed in Article I: Key Resources Table. Control siRNA was from Qiagen, and other siRNAs were manufactured by Integrated DNA Technologies. The efficiency of the knockdowns was evaluated by Western blotting or RT-qPCR assays (Article I).

9.7. RNA extraction and RT-qPCR analysis

80% confluent HeLa or HCT116 cells grown on 6-well plates were treated with specified drug combinations or 0.1% DMSO. TRI Reagent (Sigma) was used to extract RNA from the cell pellets. The residual DNA was removed using the Turbo DNA-Free kit (Thermo Fisher Scientific). To obtain cDNAs from the extracted RNA, M-MLV reverse transcriptase (Thermo Fisher Scientific) treatment was performed using random hexamers (Thermo Fisher Scientific) according to the manufacturer's instructions. qPCR reactions were performed with diluted cDNAs using primer pairs spanning the exon-intron junction of tested genes. qPCR was performed with FastStart Universal SYBR Green QPCR Master (Rox) from Sigma using Stratagene Mx3005 qPCR machine or Roche LightCycler 480. Primers were designed using PrimerQuest Tool and obtained from Integrated DNA Technologies. Relative expression of transcripts was quantified using the MxPro QPCR Software v4.10. GAPDH mRNA values were used to normalize levels of RNA expression. At least three independent experiments were performed, and results were presented as the mean ± s.e.m. The primers sequences used for RT-qPCR assays are listed in Article I: Table S5.

9.8. Cytotoxicity, cell viability, and apoptosis assays

HeLa Flp-In, RPE-1, HFF-1, and HCT116 cells were seeded on 96-well plates 16 h before the experiment to ensure 80% confluency. Concentrations of chronic treatments with the drug combinations are indicated in Article I.

Cytotoxicity and cell viability were evaluated using CellTox Green Cytotoxicity (CTxG) and CellTiter Glo (CTG) Assays (Promega) (Crouch et al., 1993). CellTox Green Dye (McDougall and Dwight, 2010) was added to the cells together with chemicals or immediately after UV irradiation. Fluorescence, in the case of CTxG and luminescence in the case of CTG assay, were measured using PerkinElmer Victor X3 reader according to the protocols

given by assay supplier at the indicated time points. Three independent experiments were shown as fluorescence values relative to the untreated control and plotted as the average ± s.e.m. The cell viability of HeLa cells was tested with the assistance of alamarBlue Cell Viability Assay (Thermo Fisher Scientific). For that, the medium containing the alamarBlue reagent was added to the cells two hours before the indicated time points. Fluorescence was then measured using PerkinElmer Victor X3 Reader. Three biological replicates are presented as fluorescence values relative to the untreated control and plotted as the mean ± s.e.m.

HeLa Flp-In and RPE-1 cells were seeded in 96-well plates 16 h before the experiment ensuring 80% confluency at the time of experiment start and subjected to the same experimental conditions as in cytotoxicity assays. Activation of apoptosis was assessed using Real Time-Glo Annexin V Apoptosis and Necrosis Assay (Promega). In this assay, PerkinElmer Victor X3 Reader was used to read luminescence at the indicated time points. Results from three independent experiments are quantified as luminescence values relative to the untreated control and mean ± s.e.m is plotted.

9.9. Co-immunoprecipitation and immunoblotting

HEK 293 Flp-In T-REx cells were grown on 10 cm cell culture dish. The cells were treated for 16h with 1 μg/ml tetracycline to express the 3XFLAG epitope-tagged proteins. The cells were exposed to DMSO or 5 M 4-NQO for different times. Whole-cell extracts (WCE) were prepared by resuspension of the cell pellets in lysis buffer C (20 mM Tris-HCl, 0.5% NP-40, 150 mM NaCl, 1.5 mM MgCl2, 10 mM KCl, 10% Glycerol, 0.5 mM EDTA, pH 7.9) on ice for 15 min in the presence of EDTA-free Protease Inhibitor Cocktail (Sigma). For FLAG immunoprecipitation, WCE were incubated at 4°C for 4 h with anti-FLAG M2 affinity gel (Sigma) equilibrated with buffer C. Purified protein complexes were washed three times with lysis buffer C, eluted by heat-treatment at 95°C in SDS running buffer with 10% of 2-mercaptoethanol (Sigma) for 5 min and separated in 10% SDS-PAGE. For other immunoprecipitation experiments, 1-2 μg of antibodies with protein complexes were immobilized on Protein G Dynabeads (Thermo Fisher Scientific).

9.10. Immunofluorescence microscopy

HeLa cells were grown on poly-lysine coated coverslips. The cells were treated with DMSO or 4-NQO for 1 h, washed with PBS, and incubated in the cytoskeletal buffer (CSK) for 10 min. Cells were then fixed in CSK buffer (10 mM Pipes pH 6.8, 300 mM sucrose, 100 mM NaCl, 3 mM MgCl2, 1 mM EGTA) supplied with 4% formaldehyde for 1 h on ice and blocked with TBS-I for at least 1 h on ice. Primary antibody staining was done overnight at 4°C, which was followed by two washes with ice-cold CSK buffer. Secondary antibody staining was performed for 4 h at 4°C. Cells were then washed twice with ice-cold CSK buffer and incubated for 30 min at room temperature in the presence of NucBlue reagent (Thermo Fisher Scientific) followed by washing in ice-cold CSK buffer. Next, coverslips were mounted using ProLong Gold Antifade Mountant (Thermo Fisher Scientific). Images were acquired using the AxioLab system microscope. AxioVision 4.3 Microscopy Software (Zeiss) and CorelDRAW Graphic Suite 2017 package were used to analyze the images

The cells with at least one γ-H2AX aggregate from two independent experiments were counted, plotted as a percentage of the total number of cells in the field, and presented as the mean ± s.e.m. Cells from at least ten fields containing at least ten cells per field were counted for each independent treatment.

9.11. iCLIP-seq assay

HEK 293 Flp-In T-REx cells were treated for 24 h with 2 μg/ml of doxycycline (Sigma) to express FLAG-tagged RBM7. Cells were exposed to DMSO or 5 μM of 4-NQO for 2 h, followed by a wash with PBS. Cells were radiated with UVC (λ=254 nm) with a dose of 150 mJ/cm^2.

An extraction of RBM7-bound RNAs was performed and sequencing libraries were prepared as described previously (Huppertz et al., 2014). The reads were aligned to the human genome (hg19) using STAR aligner within the iCount package (http://icount.biolab.si/), and cross-linked sites were defined as described previously (König et al., 2010). iCLIP experiment was performed in two biological replicates. Positions of cross-linked sites were compared between samples and then combined into groups for final analysis using iCount.

9.12. Quantitative RNA immunoprecipitation assay

HEK 293 Flp-In T-REx cells were grown on 15 cm plates. Tetracycline was added to the cells at a final concentration of 1 µg/ml. Cells were incubated for 16 h to express the 3XFLAG epitope-tagged proteins. Then, cells were exposed to DMSO or 10 µM of 4-NQO for 2 h. The cells were cross-linked with 1% formaldehyde at room temperature for 10 min in Falcon tubes with 10 ml of ice-cold PBS. Crosslinking was stopped with 250 mM of glycine for 5 min at room temperature. The RNA-protein complexes were co-immunoprecipitated using antibody-coupled magnetic beads. RNA extraction was performed using TRI reagent. The RNA was reverse transcribed. qPCRs were performed as described in Article I.

9.13. Quantitative chromatin immunoprecipitation assay

The ChIP-qPCR assay was performed as described previously (Ekumi et al., 2015). Briefly, HeLa Flp-In cells were grown on 15 cm plates to approximately 90% confluency. Then, the cells were treated for 2 h with DMSO or 5 µM 4-NQO. The cells were lysed in 800 µl of RIPA buffer and cross-linked using formaldehyde. Lysates were then sonicated at 4^{o}C. After centrifugation at 13 000xg for 15 min, 2.5% of the cleared chromatin was collected and stored at -80^{o}C for determining DNA input. The remaining insoluble fractions were supplemented with 600 µl of RIPA buffer and incubated overnight at 4^{o}C with antibody-coupled protein G Dynabeads (Thermo Fisher Scientific). The immobilized crosslinked protein-DNA complexes were washed in accordance with the protocol described in detail in Article I. DNA was purified with Phenol-Chloroform-Isoamyl (Sigma).

The purified DNA samples were PCR-amplified using primer pairs spanning genomic sequences of the selected genes with FastStart Universal SYBR Green QPCR Master (Rox) (Sigma) using LightCycler 480 II (Roche) machine. Primers were designed using PrimerQuest (Integrated DNA Technologies). All results are mean ± s.e.m from the three independent experiments. The primers used in the assay and their genomic locations are listed in Article I: Table S20.

9.14. Glycerol gradient sedimentation analysis

HeLa cells were grown to 80% confluency and treated with DMSO or 10 μM of 4-NQO for 2 h. Cells were lysed in buffer B (20 mM HEPES, 0.3 M KCl, 0.2 mM EDTA, 0.1% NP-40, 0.1% protease inhibitor, pH 7.9) on ice for 15 min. Cell lysates were subjected to ultracentrifugation in the SW41 Ti rotor (Beckman) at 38 000 rpm for 16 h in a 10 ml glycerol gradient solution (10–30%) containing buffer B. Fractions were collected and examined as described previously (Yik et al., 2003).

9.15. Bimolecular fluorescence complementation assay

Bimolecular fluorescence complementation assay was performed according to the protocol described (Fujinaga et al., 2015). Briefly, HeLa Flp-In cells were co-transfected with 0.2 μg of pEF.YN-CTD and 2 μg of pEF.YC-P-TEFb plasmids using X-tremeGENE transfection reagent (Sigma). After 1 day, the cells were seeded to 24-well plate and grown for 24 h to 48 h. The cells were then treated with DMSO, with 2, 5, and 10 μM of 4-NQO for 0.5h, 1 h, and 4 h, and with 5 μM of SAHA or JQ1 for 1 h. Fluorescence signals were detected using an Olympus IX70 fluorescent microscope. The fluorescence images were analyzed using MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices). YFP-positive cells were counted manually. An average of three randomly chosen fields of each sample was calculated.

9.16. P-TEFb release assay

P-TEFb release assay was performed as described in (Calo et al., 2015) with modifications explained in detail in Article I. Briefly, the 108 HEK 293 Flp-In T-REx F-RBM7 cells were treated with 1 μg/ml of tetracycline for 16h to express the F-RBM7 proteins. Cell extracts were prepared on ice by lysing the HeLa cells in 1.2 ml of buffer C supplied with EDTA-free Protease Inhibitor Cocktail (Sigma) on ice. 7SK snRNP anti-HEXIM1 antibody (Everest Biotech) and Protein G Dynabeads, as well as Anti-FLAG Affinity Gel F-RBM7 (Sigma), were used for purification of XXX. Immuno-purified proteins were incubated with equal amounts of immobilized 7SK snRNP for 2 h on the ice, which was

followed by the collection of samples for Western blotting analysis and RT-qPCR analysis. To examine the purity of proteins, samples were subjected to SDS-PAGE and immunoblotting with anti-hMTR4, anti-EXOSC3, and anti-CDK9 antibodies.

9.17. 4sU-sequencing of nascent RNA

RNA labeling and isolation were performed, as described previously (Rädle et al., 2013). Briefly, Hela Flp-In cells were grown to 80% confluency and then treated with DMSO, 5 μM 4-NQO, or 5 μM 4-NQO and 250 nM flavopiridol. After 0.5 or 1.5 h, the cells were labeled by adding 4-SU to the final concentration of 100 μM and incubated for 30 min more. Cells were lysed, and RNA was extracted using TRI reagent (Sigma) following the manufacturer's protocol. 150 μg of total RNA was biotinylated with EZ-Link Biotin-HPDP (Thermo Fisher Scientific) for 90 min at room temperature. The second round of RNA extraction was performed with chloroform-isopropanol. μMACS Streptavidin Kit (Miltenyi) was used for the separation of labeled RNA. Purified nascent RNA was eluted using DTT and extracted with isopropanol. Libraries from two biological replicates of the experiment were prepared and sequenced using Illumina TruSeq Platform by Beijing Genomics.

9.18. Analysis of 4sU-sequencing data sets

Sequencing quality control was performed using FastQC, and sequencing adapters were trimmed using Cutadapt (Martin, 2011). Reads were aligned against the human reference genome (GRCh38/hg38) and rRNA sequences using ContextMap 2 (Bonfert et al., 2015). FeatureCounts was employed to get read counts for mRNAs, lincRNAs, uaRNAs, and eRNAs (Liao et al., 2014).

Annotations for mRNA and lincRNA were taken from Gencode version 25. eRNAs and uaRNAs annotations were taken from the study by Lubas et al. (Lubas et al., 2011). eRNAs which are situated within 5 kbp from an annotated gene (according to Gencode) were excluded. uaRNAs were defined as the RNA within 3 kbp to the TSS of mRNA and lincRNA genes on the opposite to given mRNA or lincRNA DNA strand. If another annotated by the Gencode gene was present up to 10 kbp upstream to given TSS uaRNA was excluded from the analysis.

Expression of mRNAs, uaRNAs, eRNAs, and lincRNAs was quantified as fragments per kbp of exons per million mapped reads (FPKM) and the average

value of three biological replicates was presented. Differential gene expression analysis to determine gene expression changes and the significance of these changes was performed using edgeR (Robinson et al., 2010). RNAs with an average read count of less than one were excluded from this analysis.

p-values from edgeR calculations were corrected by multiple testing using Benjamini and Hochberg method to adjust the false discovery rate (FDR), and a p-value cut-off of 0.01 was used. Spearman rank correlation was used to find correlations between the samples. Gene lengths were obtained from the Gencode annotations.

Supplementary tables 3–6 of Article I contain mRNAs, lincRNAs, uaRNAs, and eRNAs, which were differentially expressed upon 1 h and 2 h of 4-NQO treatment. Additionally, the RNAseq data used in this study were deposited into Expression Omnibus (GEO) repository (NCBI) under the accession code GEO: GSE110272.

9.19. Ingenuity pathway and molecular signature database analysis

Ingenuity Pathway Analysis (IPA) was used to identify upstream regulators of genes expressed or inhibited after treatment with 4-NQO. The 4FP gene set was also used for IPA Downstream Effects analytic to identify the changes in biological functions in accordance with differential gene expression data. Information about these analyses could be obtained from IPA. 4FP gene set was also compared to the Molecular Signatures Database (MSigDB) collection v6.0 data sets using the Broad Institute online tool to find possible overlaps.

9.20. Transcription factor binding motifs analysis and p53 target gene enrichment analysis

RcisTarget version 1.0.2 (Aibar et al., 2017) was used to analyze the 4FP gene set to predict TF binding sites. We used gene sequences 5 kbp upstream or downstream to the TSSs (motif collection version: mc9nr). Genes, as well as transcription factors, were annotated and ranked to each motif with the normalized enrichment score (NES; cut-off of > 3). To identify p53 target genes enrichment in 4FP data set 4 gene sets from the earlier studies were used. For the genes identified in the Supplemental File S6 of the first study (Andrysik et al., 2017) both a complete set of p53 target genes (p53 – 1) as well as "core p53 target genes" were used (p53-2). We also evaluated p53 target

genes (p53-3) and set of human genes that contained p53 bound near the promoter (p53-4) from the Supplementary Table S1 of independent study (Kalan et al., 2017). An exact Fisher's test was applied for enrichment and significance of enrichment determination. To correct multiple testing the method by Benjamini and Hochberg (Benjamini and Hochberg, 1995) was used

9.21. Quantification and statistical analysis

To identify differentially expressed genes from the 4sU-seq data edgeR package for RNAseq analysis was employed (Robinson et al., 2010). Analysis results (p-values) were corrected by multiple testing using Benjamini and Hochberg's approach to adjust the false discovery rate (FDR) and with p-value cut off 0.01. All experiments were performed in at least three biological replicates. Results of qPCR-based experiments and functional assays are presented as means ± s.e.m. Statistical significance of these findings and p-values were determined using one-tailed Student's t-test performed between the indicated paired groups of biological replicates. p-values are not shown in the case of an absence of statistical significance.

10 RESULTS

10.1 DNA damage triggers the interaction of RBM7 with 7SK snRNP

Previous work has shown that RBM7 is essential for cell survival under genotoxic stress induced by DNA damaging agents (Blasius et al., 2014). However, how RBM7 promotes cell survival under genotoxic stress remains unclear. As a part of the NEXT complex, RBM7 binds to the several classes of short-lived ncRNAs, for example, uaRNAs, leading to the nuclear exosome assembly and subsequent nucleolytic degradation of the substrates (Lubas et al., 2011). Interestingly, treating the cells with 4-NQO to induce bulky UV-like DNA lesions (Ikenaga et al., 1977) promotes the p38 MAPK pathway activation leading to phosphorylation of RBM7 by MK2 (Blasius et al., 2014; Tiedje et al., 2015). Therefore, RBM7 dissociates from uaRNAs resulting in the accumulation of RNAs in the treated cells. Critically, these previously described mechanisms have not elaborated on the mechanism of cell sensitization to DNA-damaging agents upon RBM7 depletion (Blasius et al., 2014).

We hypothesized that under genotoxic stress RBM7 could exchange its ncRNA partner which would lead to cell survival. To identify the RNAs bound to RBM7 in normal and DNA damage conditions we performed RNA crosslinking immunoprecipitation followed by sequencing (iCLIP) in HEK 293 Flp-In T-Rex cells expressing 3XFLAG-tagged F-RBM7 protein. We induced DDR in these cells using the 4-NQO treatment for two hours. As expected, 4-NQO activated DDR, inducing γ-H2AX foci formation (I: Figure S1C). The protein-RNA complexes in the treated cells were then UV-crosslinked, followed by RBM7 immunoprecipitation and recovery of RNA for sequencing according to the iCLIP protocol (Huppertz et al., 2014). Consistently with previous data (Lubas et al., 2015), we found that RBM7 bound directly to the diverse types of RNAs, pre-mRNAs, and ncRNAs (I: Figures 1A, S1A and S1B, and Table S1A). Interestingly, some snRNAs, including the top 7SK, increased the binding to RBM7 in 4-NQO-treated cells in comparison to DMSO-treated cells, whereas pre-mRNAs and uaRNAs decreased the binding (I: Tables S1B and S1C).

Given that 7SK is a core of the 7SK snRNP complex and involved in Pol II transcription regulation, we focused our efforts on investigating the hypothetic role of RBM7-7SK interaction in reshaping of transcription upon genotoxic insult. According to our iCLIP data RBM7 binds 7SK selectively at the stem-loop 3 (SL3) (I: Figure 1B) in between MePCE/HEXIM1 and LARP7, that bind SL1 and SL4, respectively (Quaresma et al., 2016). We validated our findings

using RIP-qPCR experiments in HEK 293 Flp-In T-Rex cells and HEK 293 expressing RNA-binding deficient RBM7. Here, we substituted the conserved RNA-binding residues Lys60, Phe62 and Phe64 residues of the ribonucleoprotein 1 (RNP1) motif of RBM7 to Ala. Importantly, mRNP1 RBM7 did not interact with 7SK snRNA (I: Figure 1C). Moreover, while binding of the wild-type RBM7 to 7SK was increased in response to 4-NQO, the interaction with uaRNA of the RBM39 gene was decreased, which is in agreement with previous reports of the DNA damage-induced release of uaRNAs from NEXT (Blasius et al., 2014; Tiedje et al., 2015) (I: Figure S1E).

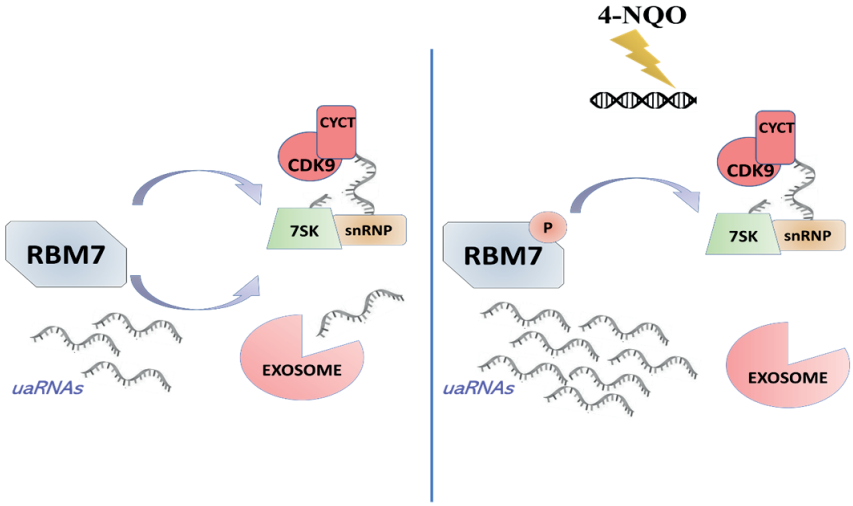

Figure 5. Schematic representation of RBM7 the RNA-interactome dynamics after DNA damage. Left: During normal cell cycle RBM7 as part of NEXT complex binds various classes of RNAs (uaRNAs are shown as an example) to drive nuclear exosome degradation of these RNAs. Right: After DNA damage induced by 4-NQO RBM7 dissociates from uaRNAs and associates with 7SK snRNA.

To complement our RIP-qPCR experiments, we performed coimmunoprecipitation (coIP) experiments with F-RBM7 and found that RBM7 binds to the LARP7 and CDK9 subunits of 7SK snRNP while it does not interact with HEXIM1 (I: Figure 2A). Thus, we identified RBM7 as a novel 7SK-interacting protein, which facilitates the cellular response to DNA damage via interaction with 7SK snRNP (Figure 5).

10.2. RBM7 releases P-TEFb from 7SK snRNP in response to DDR signaling

Since UV exposure disintegrates the 7SK snRNP (Napolitano et al., 2010) we tested if the treatment of cells with 4-NQO also affects the complex integrity. Firstly, we immunoprecipitated FLAG-tagged LARP7 component of the 7SK snRNP in HEK 293 Flp-In cells followed by immunoblot analysis to reveal that the exposure to 4-NQO resulted in the release of CDK9 and HEXIM1 from the 7SK snRNP (I: Figure 2B). Secondly, glycerol gradient centrifugation analysis showed the transition of P-TEFb into the low molecular weight fraction, which corresponds to the P-TEFb that is not incorporated into 7SK snRNP (I: Figure S2B). Finally, 4-NQO treatment diminished the interaction of 3XFLAG epitope-tagged CDK9 with 7SK snRNP components, while the interaction of P-TEFb with RBM7 increased (I: Figure 2C).

The above findings pointed out the unexpected correlation between DDR-induced interaction of RBM7 with 7SK snRNP and the release of P-TEFb from this inhibitory complex, suggesting that RBM7 may be involved in remodeling of the 7SK snRNP complex. To define a possible role of RBM7 in P-TEFb release from 7SK snRNP, we did a siRNA-mediated knockdown of *RBM7* in the HEK 293 cells expressing FLAG-tagged HEXIM1 component of the 7SK snRNP and then treated the cells with 4-NQO or DMSO. As expected, 4-NQO induced a rapid decrease in CDK9 binding to HEXIM1 in the cells treated with control siRNA, which we concluded based on the FLAG-HEXIM1 coIP. Critically, this effect was diminished in *RBM7* knockdown cells (I: Figure 3A). Interestingly, tetracycline-induced over-expression of wild-type, but not the mRNP1-mutated, RBM7 resulted in decreased interaction of HEXIM1 with CDK9 and 7SK in HEK 293 cells (Figure 3B). This observation suggested that RBM7 may compete with HEXIM1 for the 7SK binding in this model.

We next tested if the p38 MAPK signaling pathway, which triggers phosphorylation of RBM7 in response to UV irradiation (Blasius et al., 2014; Borisova et al., 2018), is involved in the release of P-TEFb from 7SK snRNP. Here, pharmacological inhibition of p38 kinase with inhibitor SB203580 prior to 4-NQO exposure prevented the RBM7 interaction with 7SK (I: Figure 3D) as well as the release of CDK9 from HEXIM1 in HeLa cells (I: Figure 3C). Thus, these findings showed that RBM7 and p38 MAPK are critical for the genotoxic stress-induced release of CDK9 from 7SK snRNP.

Since RBM7 is a part of the NEXT complex it may trigger the interaction of 7SK with the nuclear exosome followed by its degradation and the 7SK snRNP complex dissociation. To test this hypothesis, we first depleted the two other NEXT subunits ZCCHC8 and MTR4, which link RBM7 with the core exosome (Falk et al., 2016; Lubas et al., 2011), in HeLa cells, followed by 4-NQO treatment. We found that knockdowns of the *ZCCHC8* and *MTR4* do not

prevent the exit of CDK9 from the complex with HEXIM1 upon DNA damage (I: Figure S3A). Moreover, kinetic FLAG coIP (I: Figure 4A) and RIP-qPCR (I: Figure 4B) confirmed that the 4-NQO-induced release of CDK9 and HEXIM1 from F-LARP7 left the core of 7SK snRNP, consisting of 7SK snRNA, MePCE, and LARP7, intact. In addition, 4-NQO treatment triggered the inclusion of hnRNP A1 into the complex with F-LARP7 (I: Figure 4A). Of note, hnRNP A1 and other hnRNPs were shown to replace P-TEFb and HEXIM1 within 7SK snRNP upon Pol II inhibition by binding SL3 of 7SK (Barrandon et al., 2007; Van Herreweghe et al., 2007). Finally, we observed no changes in total 7SK snRNA levels upon 4-NQO treatment (I: Figure 4B). In conclusion, our data demonstrate the integrity of the core 7SK snRNP complex during 4-NQO exposure, arguing against a possibility that the RNA exosome degrades 7SK following genotoxic stress.

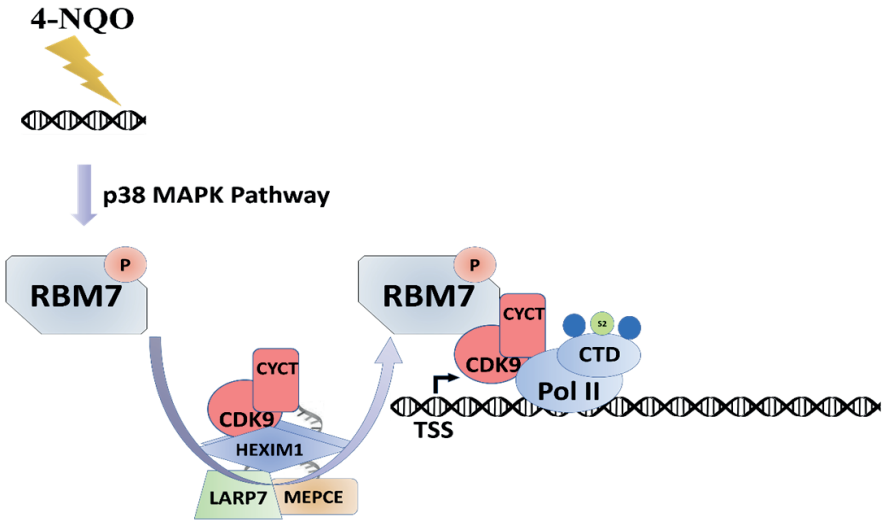

Figure 6. A simplified model of DDR-induced P-TEFb release by RBM7. The cartoon showing the sequence of events in the RBM7-P-TEFb pathway: 4-NQO-induced activation of DDR through the p38 MAPK pathway, which leads to the release of P-TEFb from 7SK snRNP by RBM7.

We examined if RBM7 could act directly on 7SK snRNP as a novel P-TEFb release factor (Quaresma et al., 2016). To test the RBM7 competence to remodel the 7SK snRNP complex, we performed a P-TEFb release from the 7SK snRNP assay (Calo et al., 2015) *in vitro* (I: Figure 4C). The 7SK snRNP complex from HeLa cells was first immobilized on the magnetic beads using an antibody to the endogenous HEXIM1 followed by incubation with the increasing amounts of immunopurified RBM7 protein diluted in the lysis buffer. Upon incubation, the magnetic beads with 7SK snRNP bound through the HEXIM1 antibody were subjected to immunoblot and RT-qPCR analyses. We found that RBM7 is capable of releasing CDK9 and 7SK from the complex

with HEXIM1 protein *in vitro*, while the mRNP1 F-RBM7 failed to do so (I: Figure 4D).

We next purified the wild-type and mRNP1-mutant RBM7 proteins tagged with maltose-binding protein from E. coli (MBP-RBM7) to perform *in vitro* binding experiments with 7SK snRNP components. Glutathione-S-transferase (GST)-tagged HEXIM1, LARP7, and His-tagged CDK9 full-length proteins, as well as the catalytic domain of MePCE (cMePCE; residues 400-689) and cyclin-box domain of CycT1 (residues 1-272), were manufactured in E. coli and served as models of native proteins (I: Figure S3C). Here, MBP-RBM7 of wild type, in contrast to RNA binding-deficient mRNP1 MBP-RBM7 mutant, interacted with GST-cMePCE and GST-LARP7 (I: Figures 4E and 4F, S3D). In contrast, GST-HEXIM1 failed to interact with MBP-RBM7 in agreement with the data from whole-cell extracts (I: Figures 4G and S3E) suggesting that RBM7 binds 7SK snRNP mutually exclusive to HEXIM1.

In sum, the above findings proved that in the 4-NQO-challenged cells RBM7 interacts directly with 7SK and subunits of 7SK snRNP to release P-TEFb from the inhibitory HEXIM1-containing complex (Figure 6).

10.3. P-TEFb phosphorylates Pol II to induce a transcriptional response to the genotoxic insult

We next studied the functions of P-TEFb, released from 7SK snRNP by RBM7, in 4-NQO-induced DDR. Importantly, upon the 4-NQO treatment, FLAG-tagged CDK9 and RBM7 increased binding to the transcriptionally active Ser2-P form of Pol II (I: Figures 2C and 2D). We visualized the relocation of P-TEFb to the CTD of Pol II by conducting Visualization of P-TEFb Activation (V-PAC) assay (Fujinaga et al., 2015). In HeLa cells, we transiently expressed YC-P-TEFb and YN-CTD chimera containing the C- and N-terminal regions of yellow fluorescent protein (YFP) respectively (I: Figure 2E). In agreement with co-IP data, we observed an increased number of YFP-positive cells upon 4-NQO exposure, which indicates an increased interaction between YC-P-TEFb and YN-CTD (I: Figures 2F and S2F). The latter result suggested that genotoxic stress triggers the relocation of P-TEFb from 7SK snRNP to the CTD of Pol II.

To confirm that the P-TEFb release from 7SK snRNP and the subsequent relocation to Pol II results in a productive Pol II transcriptional response to 4-NQO treatment, we performed nascent RNA sequencing in HeLa cells treated with DMSO or 4-NQO (I: Figure 5A). We labeled newly transcribed RNA using 4-thiouridine (4sU) during the treatments, followed by total RNA extraction,

biotinylation, and separation of nascent RNA on streptavidin beads and sequencing (4sU-seq) (Rädle et al., 2013). To dissect the role of P-TEFb in the Pol II transcription in response to DDR we used CDK9 inhibitor flavopiridol in parallel with 4-NQO treatment for 4sU-seq samples preparation (Chao et al., 2000).

As a result of the differential gene expression (DE) analysis of the 4sU-seq libraries, we found that the transcription of the majority protein-coding genes was down-regulated upon 4-NQO treatment. In contrast, most of the affected ncRNAs, as well as a fraction of the mRNA-encoding genes, were up-regulated (I: Figures 5A and 5B) (Figure 7). Importantly, the transcriptional changes were highly dependent on active P-TEFb since flavopiridol co-treatment diminished transcriptional response (I: Figure 5B). Also, we found that the up-regulated mRNA coding genes were considerably shorter than the down-regulated genes (I: Figure 5C). This observation agrees with the downregulation of the elongation of long genes previously described for the transcriptional response to UV treatment (McKay et al., 2004; Williamson et al., 2017).

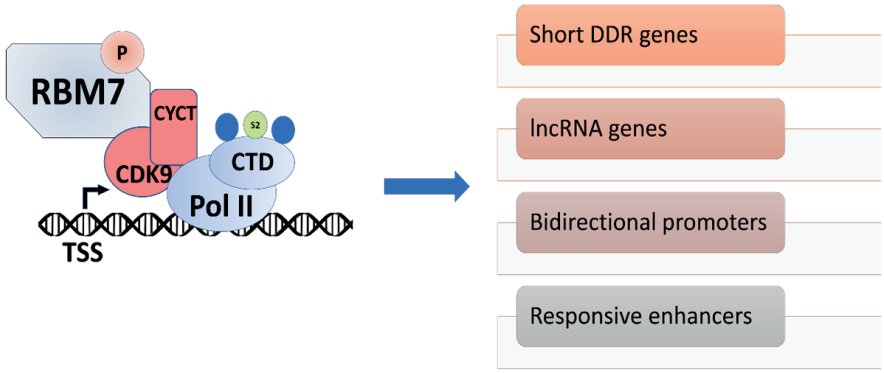

Figure 7. Main classes of the P-TEFb targets after 4-NQO treatment according to the nascent transcriptome data. The cartoon depicting the released P-TEFb together with elongating Pol II and the classes of transcription units affected by P-TEFb activation.

In order to put the activation of ncRNAs, such as uaRNAs and eRNAs, into the functional context, we studied the relationships of their expression with the expression of neighboring protein-coding genes. Here, to dissect if the accumulation of uaRNAs was a result of the loss of transcription directionality of the bidirectional promoters rather than activation of specific uaRNAs we first paired uaRNAs to mRNAs originating from the same promoter. Then the numbers of reads per kilobase of transcript per million mapped reads (RPKM values) were calculated for both uaRNA and corresponding mRNA and plotted one against another. We found a weak correlation between uaRNA and the host gene expression in the non-treated cells ($r = 0.31$), whereas after 1 h and 2 h of 4-NQO treatment the correlation of RPKM values within the uaRNA-

mRNA pairs increased (r = 0.44, r = 0.47, respectively). Inhibition of 4-NQO-induced transcription response using FP eliminated the observed effect of 4-NQO on these relationships (Figure 8). Based on this analysis, we concluded that the upregulation of uaRNAs upon 4-NQO treatment was a result of P-TEFb-mediated activation of bidirectional transcription from the host gene promoters.

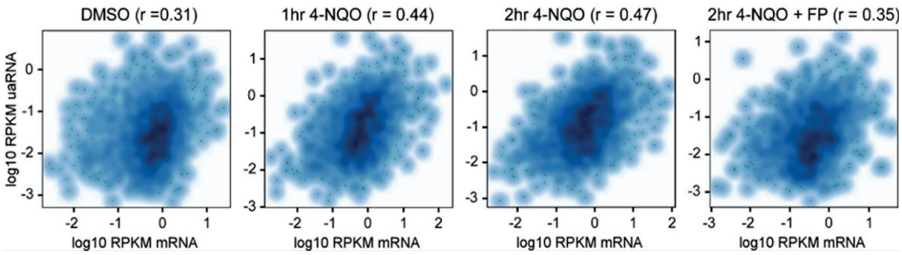

Figure 8. Changes of uaRNA-mRNA pairs transcription around bidirectional promoters. Changes of RPKM values in 4sU-seq of individual mRNA-uaRNA pairs plotted for different experimental conditions. The correlation coefficient is indicated on the top of each plot (Analysis of data: Prof. Caroline Friedel; unpublished data related to Article I).

Interestingly, 4-NQO-induced upregulation of transcription of the RBM39 mRNA, which is a product of the gene with a bidirectional promoter, was linked with the accumulation of its uaRNA (I: Figure 5E). The 4sU-seq also found that eRNAs are among the most responsive to 4-NQO classes of RNA. We specifically tested with RT-qPCR the dynamics of transcription activation of the EGR2 gene and its eRNA and found connected profiles of DDR induced activation (I: Figure 5E).

Since the observed transcriptional response to 4-NQO was highly specific and involved only a relatively small number of genes, we concluded that additional to P-TEFb transcription factors are involved in the Pol II activation. We employed the Ingenuity Pathway Analysis (IPA) database to predict those transcription factors based on our differentially expressed gene lists and identified p53 as a top upstream regulatory factor (I: Table S2F). We next created the set of 4-NQO-activated and P-TEFb-dependent genes according to the effect of flavopiridol (4FP set, Table S3A) treatment and analyzed it with the assistance of IPA and Molecular Signatures Database (MSigDB) database. 4FP set was enriched in NF-kB and p53 pathway targets (I: Table S3C) (Andrysik et al., 2017; Fischer, 2017). In addition, 4FP genes were involved in such cellular processed as death and survival, proliferation, gene expression and cell cycle (I: Figure 5D). Critically, both NF-kB and p53 have shown to act in the DDR (Gomes et al., 2006; McKay et al., 2004; Sullivan et al., 2018; Wu et al., 2006) and require P-TEFb activity for their functions (Barboric et al., 2001; Gomes et al., 2006). Also, the 4FP set showed similarity with the UV-induced gene sets of the MSigDB collection (p-value=3.8x10-50 to 2.02x10-

56; I: Table S3F) and was predicted by IPA to respond to DNA damage-inducing agents (I: Table S3G).

We next confirmed the P-TEFb-mediated activation of key DDR genes, including JUN, FOS and EGR1, a cell cycle inhibitor and a pro-survival p53 target CDKN1A/p21, an anti-apoptotic MCL1, and a DDR regulator GADD45b using kinetic qRT-PCR assays in HeLa cells treated with 4-NQO or 4-NQO in combination with FP (I: Figure 5E). Here, the inhibition of P-TEFb with FP eliminated the accumulation of selected transcripts in response to DDR. We verified if P-TEFb phosphorylates the CTD of Pol II on Ser-2 resides at these DDR genes using chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) with total Pol II, Ser2-phosphorylated (Ser-2P) Pol II and CDK9 antibodies. In support of the P-TEFb role, 4-NQO treatment-induced recruitment of CDK9 to the promoters of the tested genes. The latter coincided with the increase of the Ser2-P enrichment over total Pol II, a hallmark of elongating Pol II (I: Figures 6A and S5A). Of note, FP blocked the increase in Ser2-P levels, indicating the role of P-TEFb in stimulating Pol II transcription following 4-NQO exposure (I: Figure S5B).

10.4. RBM7-mediated release of P-TEFb from 7SK snRNP is essential for the transcriptional response to 4-NQO-induced DDR

According to our model, DDR-induced interaction of RBM7 with 7SK snRNP is essential to release P-TEFb from the complex. P-TEFb, in turn, fuels the DDR transcription program. If this is the case, interfering with RBM7, 7SK snRNP, or P-TEFb should diminish the 4-NQO-triggered gene induction. To test this model, firstly, we identified RBM7-dependent DDR genes. We performed siRNA-mediated knockdown of *RBM7* to obtain 4sU-seq data in HeLa cells treated with 4-NQO or DMSO in the absence or presence of RBM7. Surprisingly, we observed rather a moderate decrease in the number of 4-NQO-induced genes upon *RBM7* knockdown (Figure 9A). Importantly, plotting fold change values of the genes, differentially expressed in response to 4-NQO in the control HeLa cells against the values in the cells treated with RBM7 siRNA showed a strong correlation of gene expression programs in both conditions (Figure 9B). These findings suggested that, even though RBM7 knockdown had a significant impact on DDR-induced activation of the set of genes, 4-NQO-mediated accumulation of ncRNAs could be triggered by additional RBM7-independent mechanisms. Since DDR also induced dissociation of RBM7 from uaRNAs we concluded that some of the observed

4-NQO-induced accumulation of ncRNAs may be a result of the failure of nuclear RNA degradation by exosome (I: Figure 1). Nevertheless, we observed a major shift in fold-change values towards an x-axis outside of the area of high correlation, representing the transcription units with a decreased 4-NQO response upon *RBM7* knockdown (Figure 9B). Interestingly, the genes showing higher fold-changes in response to 4-NQO tended to be differentially expressed only in *RBM7* knockdown cells, representing likely gene expression changes in response to RBM7 depletion, and not to 4-NQO (Figure 9B). Hence, we decided to focus our efforts on RBM7-dependent 4-NQO-responsive genes.

We selected a set of RBM7-dependent genes and performed RT-qPCR assays in HeLa cells treated with 4-NQO. Here, the siRNA-mediated knockdown of RBM7 decreased the gene transcription activation of these DDR genes in response to 4-NQO (I: Figures 6B and S5C). Furthermore, the depletion of RBM7 diminished 4-NQO-induced upregulation of the Pol II Ser2-P mark at the transcription start sites of selected genes, which was detected using ChIP-qPCR assay (I: Figures 6B and S5C). Because RBM7 participates in the trimeric NEXT complex, we performed knockdown of other NEXT components ZCCHC8 and MTR4 to confirm that these proteins did not affect 4-NQO-triggered gene activation and RBM7 functions in this context (I: Figures S5E and S5F). In addition, we used HeLa Flp-In cell lines, which constitutively overexpress exogenous proteins of wild type and mRNP1 mutated F-RBM7, or empty FLAG-tag, to estimate DDR genes induction in the presence of 4-NQO. Interestingly, while the expression of exogenous F-RBM7 did not affect the accumulation of DDR transcripts mRNP1 mutated F-RBM7 acted in a dominant-negative fashion, resulting in poor DDR-induced transcription in those cells (I: Figure 6C).

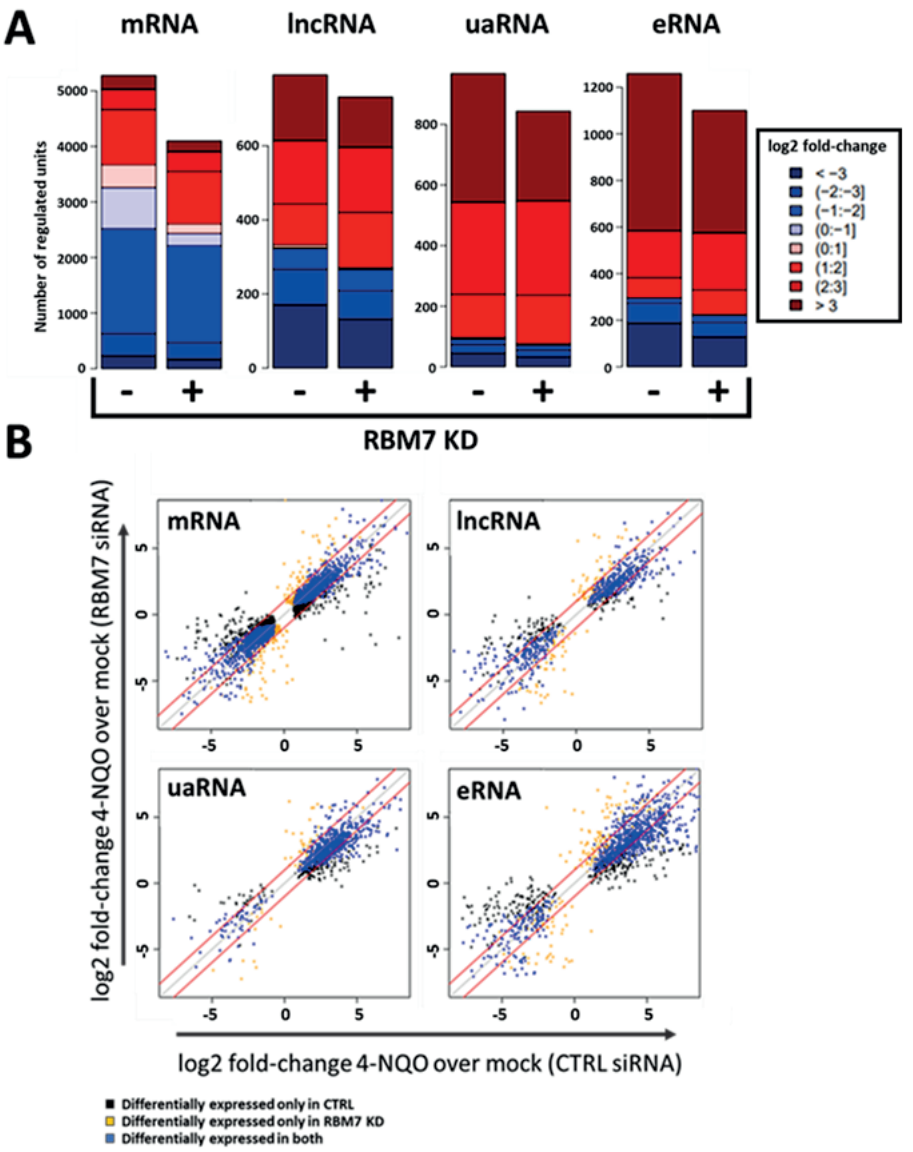

Figure 9. Effects of RBM7 knockdown on 4-NQO-induced gene expression. A. Bar graphs visualizing numbers of differentially expressed transcription units based on 4sU-seq in response to 4-NQO in cells treated with control and RBM7-targeting siRNA separated by classes of RNA. B. Dot plot with logarithmic fold-change values of differentially expressed genes upon 4-NQO treatment in comparison to DMSO-treated control. The axes represent the fold-changes in siCTRL and siRBM7 treated cells. The red lines represent an area of the high correlation between data sets ($r > 0.95$). The data is generated as a mean from two biological replicates, only statistically significant DE genes are shown (Analysis of data: Prof. Caroline Friedel; Unpublished data related to Article I).

Next, we started to test if the other steps of the proposed RBM7-P-TEFb pathway (Figure 10) have an impact on the DDR genes transcription activation after 4-NQO. We firstly inhibited the p38 MAPK pathway, which is important for 4-NQO-induced interaction of RBM7 with 7SK and P-TEFb release from 7SK snRNP, by pre-treatment of the cells with p38 inhibitor to quantify gene transcription response to 4-NQO estimated with RT-qPCR (I: Figure S5D). Indeed, the inhibition of p38 decreased DDR genes induction upon 4-NQO treatment supporting our previous conclusions about the role of p38 MAPK signaling in P-TEFb activation by RBM7.

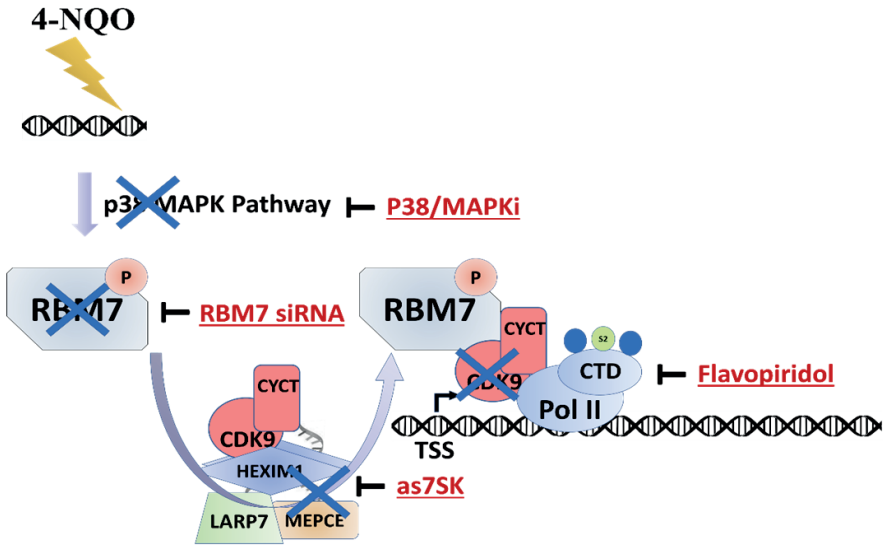

Figure 10. The points of intervention into the proposed model proving significance on DDR-induced transcription. The points of RBM7-P-TEFb pathway attenuation are shown as blue crosses. Agents used for the blockage of the specific steps are shown using red text.

Therefore, we tested if 7SK snRNP, as a reservoir of cellular P-TEFb, is essential for RBM7-mediated transcription response to DDR. To disintegrate 7SK snRNP prior to DDR activation, we triggered degradation of 7SK with specific phosphorothioate-modified antisense DNA oligodeoxynucleotides 48 h before applying 4-NQO. Here, the depletion of 7SK also decreased DDR-genes activation in response to genotoxic stress (I: Figure 6D). To conclude with our model (Figure 10), treating the cells with P-TEFb inhibitor FP completely diminished activation of the selected DDR target genes by 4-NQO (I: Figure 5E). Taken together our mechanistic experiments, we have confirmed that P-TEFb released by RBM7 from 7SK snRNP is responsible for the specific gene transcription response to 4-NQO treatment.

10.5. The P-TEFb-mediated transcriptional program promotes cell survival during DDR

Due to the importance of RBM7-mediated release of P-TEFb from 7SK snRNP for activation of Pol II transcriptional response to 4-NQO, we next studied if this mechanism is important for the cell functions. Indeed, the IPA Downstream Effects Analysis tool indicated that many 4FP genes, which were activated by 4-NQO and inhibited by flavopiridol, promote cellular viability (I: Table S3H). To prove the findings of data analyses experimentally, we performed several cell-based assays to check cytotoxicity, cell viability, and apoptosis rates in cells challenged with 4-NQO. We used Cell Tox Green cytotoxicity assay (CTxG) to estimate the kinetics of cytotoxicity in HeLa, human retina epithelium (RPE-1) and human foreskin fibroblasts (HFF-1) cell lines treated with 4-NQO only or in combination with P-TEFb inhibitor flavopiridol and found that such co-treatment sensitized cells to 4-NQO (I: Figures 7A and S6A). Of note, pre-treatment with flavopiridol triggered also hypersensitivity of RPE-1 cells to UV irradiation (I: Figure S6C).

Given the earlier data about the RBM7 requirement for cell survival in DNA damage condition (30) and our data about involvement of RBM7 in DDR-induced P-TEFb activation, we decided to test if depletion of RBM7 will sensitize cells to 4-NQO in our system. Importantly, knockdown of *RBM7* using two different siRNAs in both HeLa and RPE-1 cell lines resulted in a similar hypersensitivity to 4-NQO and UV (I: Figures 7B, S6B, and S6C). Interestingly, RBM7 depletion also sensitized HeLa and RPE-1 cells also to flavopiridol, which we addressed using kinetic CTxG assays (Figure 11). Since treatment by flavopiridol inhibits and releases P-TEFb from the 7SK snRNP (I: Figure S3B), we suggested that RBM7 may have some additional functions, besides the P-TEFb activation, in the gene transcription and cell survival.

To support the above siRNA findings, we performed an additional rescue experiment. Here, using HeLa Flp-In cell lines, stably overexpressing exogenous F-RBM7 proteins we depleted specifically endogenous RBM7 with siRNA targeting 3'UTR of the RBM7 gene. Importantly, knockdown of RBM7 sensitized the cells to 4-NQO in CTxG assay in the control maternal HeLa Flp-In cells, but the expression of the exogenous F-RBM7 of wild type rescued the effect of knockdown, decreasing cytotoxicity rates. Of note, overexpression of RNP1 F-RBM7 failed to rescue the effects of depletion of the endogenous protein expression in this system, supporting the specificity of the findings (I: Figure S6D).

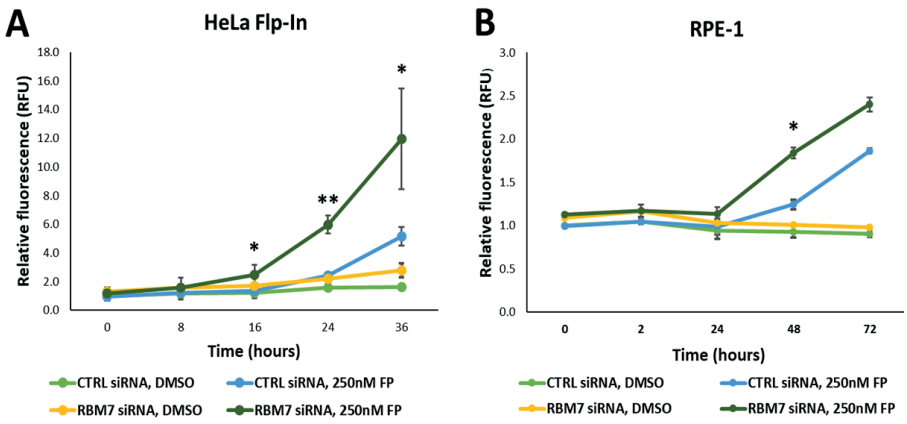

Figure 11. The depletion of RBM7 sensitizes cells to flavopiridol. Kinetic CTxG assays performed in HeLa (A) and RPE (B) cells treated with RBM7 siRNAs and flavopiridol (FP). Averages of three experiments plotted as the mean ± s.e.m. (n = 3). Statistical significance indicated by stars: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and was determined by Student's t-test. (Unpublished data related to Article I).

Corroborating our cytotoxicity results, P-TEFb inhibition, and RBM7 depletion decreased the viability of 4-NQO-exposed HeLa cells in alamarBlue cell survival assay (I: Figures S6E and S6F). Finally, we followed the kinetics of apoptosis in the cells challenged with 4-NQO and P-TEFb inhibited with flavopiridol using a luminescence-based Annexin V Assay (Dixon et al., 2016). P-TEFb inhibition, as well as RBM7 depletion, enhanced 4-NQO-stimulated apoptosis of HeLa cells (I: Figures 7C and 7D). Importantly, the onset of annexin signaling activation preceded the occurrence of cytotoxicity detected by CTxG assays, suggesting that cell death occurred through apoptosis. We confirmed the activation of apoptosis observed in Annexin V assay by the analysis of the whole HeLa cells cell lysates using immunoblotting. Here, both flavopiridol treatment and genetic knockdown of RBM7 increased 4-NQO-induced levels of cleaved poly(ADP-ribose) polymerase (PARP) and Caspase-3 (I: Figures S6H and S6I). In sum, the above findings demonstrate the critical importance of the RBM7–P-TEFb axis in enabling a pro-survival transcriptional response to genotoxic stress. Attenuation of this mechanism by either inhibiting P-TEFb or RBM7 knockdown drives the cells to the apoptotic program.

10.6. p53 directs the Pol II transcription during DDR induced by 4-NQO

Our analysis of the 4sU-seq data suggested the critical role p53 as the critical regulator of the 4-NQO-induced transcriptome (I: Figure S5H and Tables S3B and S3C). Therefore, we decided to study the impact of p53 in sensitizing the cells to the DNA damage and P-TEFb inhibition. For this, we employed human colon carcinoma cell lines HCT116 carrying wild-type TP53 (HCT116 $TP53^{+/+}$) or lacking the TP53 gene (HCT116 $TP53^{-/-}$). Here, we induced genotoxic stress by 4-NQO treatment combined with P-TEFb inhibition using flavopiridol. Such combined treatment efficiently induced cytotoxicity in HCT116 cells carrying $TP53^{+/+}$ but did not elevate cytotoxicity levels in HCT116 $TP53^{-/-}$ cells (I: Figure S6G). This observation indicated that p53 plays an essential role in the regulation of cellular response to 4-NQO-induced DNA damage and by priming the cells for P-TEFb-mediated transcription dependency.

We next studied if p53 is involved in P-TEFb-dependent gene transcription in response to 4-NQO. Of note, our data showed that p53 status was not important for the 4-NQO-induced release of P-TEFb from 7SK snRNP (I: Figure S5G) implying the involvement of p53 in targeting P-TEFb to the responsive genes. Indeed, the lack of functional p53 in HCT116 $TP53^{-/-}$ cells led to deficient transcription activation at specific genes, including CDKN1A/p21, EGR2 and FOS (I: Figure S5H) which are shown to be critical p53 effectors (Andrysik et al., 2017).

10.7. Activation of p53 by 5-FU treatment requires functional CDK9 for cancer cell survival

Given our finding of the role of P-TEFb as a pro-survival factor in DDR, we decided to study if other DDR-inducing agents may trigger the dependency on P-TEFb. In the first place, we tested if the inhibition of the CDK9 subunit of P-TEFb sensitizes HCT116 cells to chemotherapy agent 5-fluorouracil (5-FU) (Heidelberger et al., 1957). Of note, 5-FU was shown before to synergize with pan-CDK inhibitor dinaciclib, which also inhibits CDK9 (O'Neil et al., 2016). Because of the recent release of specific CDK9 inhibitors (Olson et al., 2018), we decided to use NVP-2, as a highly potent CDK9 inhibitor, for silencing of the kinase activity of P-TEFb. Here we treated HCT116 cells with combinations of NVP-2 and 5-FU to perform CTxG assay and calculate the cytotoxicity values in comparison to the non-treated control. Importantly,

NVP-2 in nanomolar concentrations had a synthetic lethal effect and predisposed the cells for death induced by 5-FU (Figure 12A) (Bugai A. et al. unpublished results).

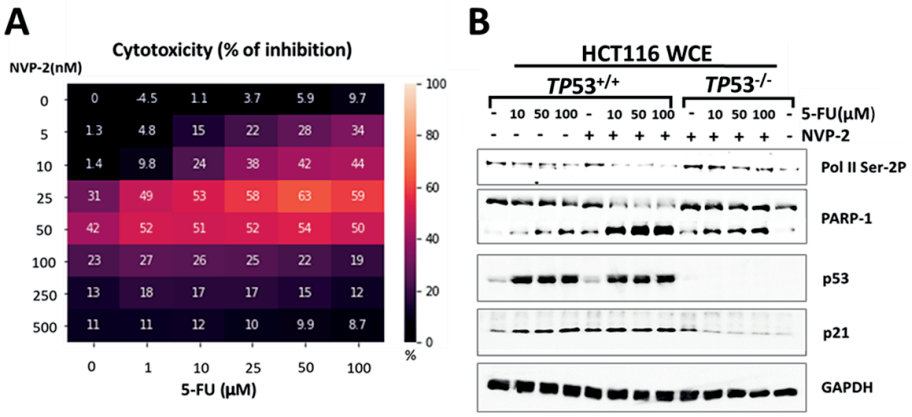

Figure 12. CDK9 inhibitor NVP-2 is synthetic lethal with 5-fluorouracil in HCT116 cells. A. Heat map representing cytotoxicity in HCT116 cells treated with combinations of NVP-2 and 5-FU for 48 h. The results of the CTxG assay presented as an average % of inhibition of 3 biological replicates. B. Immunoblot analysis of whole-cell extracts of HCT116 cells carrying TP53 of wild type (p53 +) or TP53$^{-/-}$ (p53 –) and treated with indicated drug combinations for 24 h (Bugai A. et al. unpublished results).

To confirm p53-driven apoptotic pathway activation, we did an immunoblot analysis of whole-cell extracts of HCT116 cells treated with drug combinations. Therefore, treatment with increasing amounts of 5-FU induced accumulation of p53, as well as direct p53 target p21/CDKN1A, in HCT116 cells of wild type, but not HCT116 TP53$^{-/-}$ cells (Figure 12B). 5-FU in the tested concentrations of 10-100nM induced a minor effect on PARP-1 cleavage which was estimated by total PARP-1 western blot and supported by CTxG cytotoxicity data (Figure 12A, first row).

Critically, co-treatment of the HCT116 cells carrying wild type p53 with a suboptimal concentration of CDK9 inhibitor NVP-2 (Figure 12A, first column) in addition to 5-FU dramatically increased PARP-1 cleavage (Figure 12B) indicating elevated apoptosis rates. The same combinations of NVP-2 and 5-FU had no such effect in HCT116 TP53$^{-/-}$. This data is supported by previous studies on the role of p53 in 5-FU induced cell death (Bonini et al., 2004; Krause et al., 2000; Yonish-Rouach et al., 1996). Of note, while NVP-2 decreased signal of phosphorylated Pol II Ser2-P detected by immunoblotting, the CDK9 inhibition had no effect on the accumulation of p53 and p21 upon 5-FU treatment (Figure 12B). In summary, synthetic lethality between 5-FU and NVP-2 supported the idea that P-TEFb activity is required for cell survival upon genotoxic stress. Of note, NVP-2-mediated hypersensitivity was dependent on p53 status indicating an involvement of this critical factor in the apoptosis in these conditions.

10.8. p53 accumulation in the absence of DNA damage induces CDK9 dependency of HCT116 cells

Given our data on the involvement of p53 in the transcriptional response to DDR (Figure 12; I: Figures S5G; S6G-H), we investigated if non-genotoxic activation of p53 also triggers the dependency of cancer cells on P-TEFb activity (Radhakrishnan SK, 2008). To dissect the specific role of P-TEFb in cell survival, we employed pharmacological inhibition of CDK9 with three highly selective inhibitors: the ATP-competitive i-CDK9 and NVP-2, and the thalidomide-conjugated degrader SNS032-THAL, of which THAL and SNS-032 parts bind the ubiquitin E3 ligase Cereblon (CRBN) and CDK9, respectively (Lu et al., 2015; Olson et al., 2018). We treated HCT116 $TP53^{+/+}$ and HCT116 $TP53^{-/-}$ cells with the increasing concentrations of these three different CDK9 inhibitors for 48 h to estimate the cell toxicity using CTxG assays (Figure 13). We found that CDK9 inhibitors induced a biphasic cytotoxicity response that could stem from the off-target effects of these compounds at high concentrations. Importantly, the resistance of $TP53^{-/-}$ cells to i-CDK9, NVP-2 and SNS032-THAL suggested that p53 is critical for inducing cell death upon inhibition of P-TEFb (Figure 13) (Bugai A. et al. unpublished results).

Since NVP-2 was the most potent CDK9 inhibitor we analyzed the whole-cell extracts of HCT116 $TP53^{+/+}$ and HCT116 $TP53^{-/-}$ cells by immunoblotting to verify the activity of this drug. In agreement with inhibition of CDK9, the Ser2-P Pol II signal was decreased efficiently by NVP-2 regardless of the p53 status, while the levels of total Pol II or CDK9 remained the same (Figure 14A). NVP-2 treatment also activated p53, perhaps due to inhibition of transcription (Wang et al., 2014). Remarkably, the accumulation of p53, in this case, did not result in the activation of p21/CDKN1A (Figure 14A).

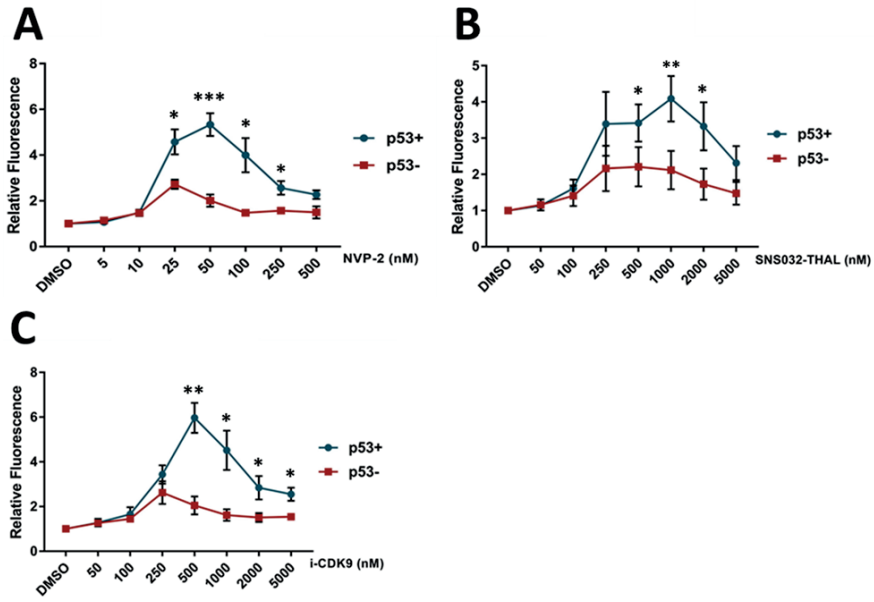

Figure 13. Cytotoxicity of CDK9 inhibitors depends on p53. Relative cytotoxicity in CellTox Green cytotoxicity assay in HCT116 $TP53^{+/+}$ (p53+) and $TP53^{-/-}$ (p53-) treated with CDK9 inhibitors: NVP-2 (A), SNS032-THAL (B) and i-CDK9 (C) for 48 h. Line charts indicate average values of fluorescence in CTxG assay relative to non-treated control and error bars represent s.e.m. The statistical significance: *p< 0.05; **p< 0.01; ***p< 0.001, determined by Student's t-test (Bugai A. et al. unpublished results).

To achieve a nongenotoxic accumulation of p53 in HCT116 cells, we employed Nutlin-3a, which perturbs the interaction of p53 with the E3 ubiquitin-protein ligase MDM2, critical for p53 degradation (Andrysik et al., 2017). Here, the treatment of HCT116 cells with increasing concentrations of Nutlin-3a led to a dose-dependent increase in the levels of p53 and p21 signals in the whole-cell extracts, confirming the activation of the p53 pathway. Finally, Nutlin-3a had no effect on Pol II or CDK9 levels in the cells, which we analyzed by immunoblotting (Figure 14B).

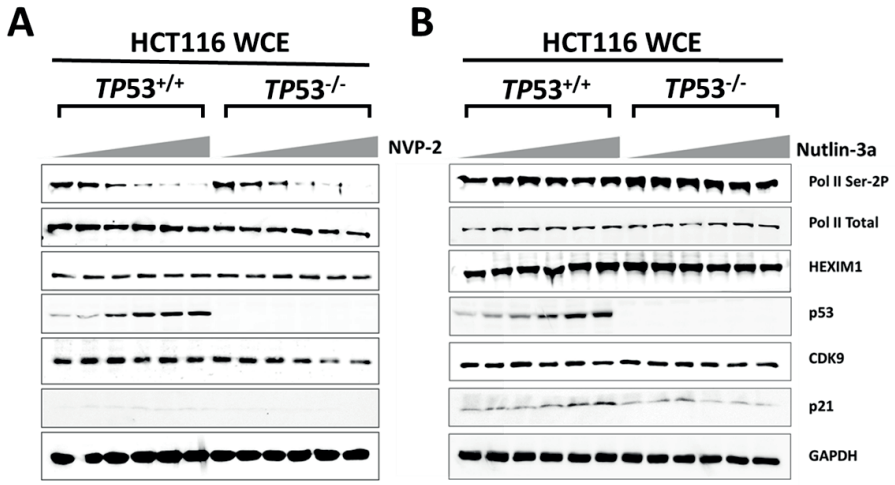

Figure 14. Analysis of NVP-2 and Nutlin-3a activity. A. Immunoblot analysis of whole-cell extracts of the HCT116 $TP53^{+/+}$ and HCT116 $TP53^{-/-}$ cells treated with increasing concentrations of NVP-2 (0, 10, 25, 50, 100, 250 nM) for 8 h. B. The same as A but for Nutlin-3a (0, 0.5, 1.25, 2.5, 5, 10 μM) (Bugai A. et al. unpublished results).

As expected, treatment with Nutlin-3a in concentrations up to 10μM was not toxic for HCT116 cells. In contrast, the combination of Nutlin-3a with NVP-2 induced cell cytotoxicity, confirming the crucial role of active P-TEFb for the survival of these cells (Figure 15A (left)). Of note, HCT116 $TP53^{-/-}$ cells are resilient to the same drug concentrations which confirm the specificity of our findings (Figure 15A (right)). We also examined cell survival in this system by employing an independent CellTiter Glo survival assay. Using the same conditions, we found that the co-treatment of the HCT116 $TP53^{+/+}$ but not HCT116 $TP53^{-/-}$ led to the decrease in cell survival (Figure 15B). In order to quantify the synergy between NVP-2 and Nutlin-3a, we calculated Bliss Synergy scores (Bliss, 1939) of the drug combinations and plotted them using SynergyFinder (Ianevski A, 2017). We found that the combination of these two compounds was highly synergistic with an average Bliss score of 8.82 (Figure 15C), meaning that the cell toxicity of NVP-2 and Nutlin-3a applied together was greater than the sum of their separate effects at the same doses.

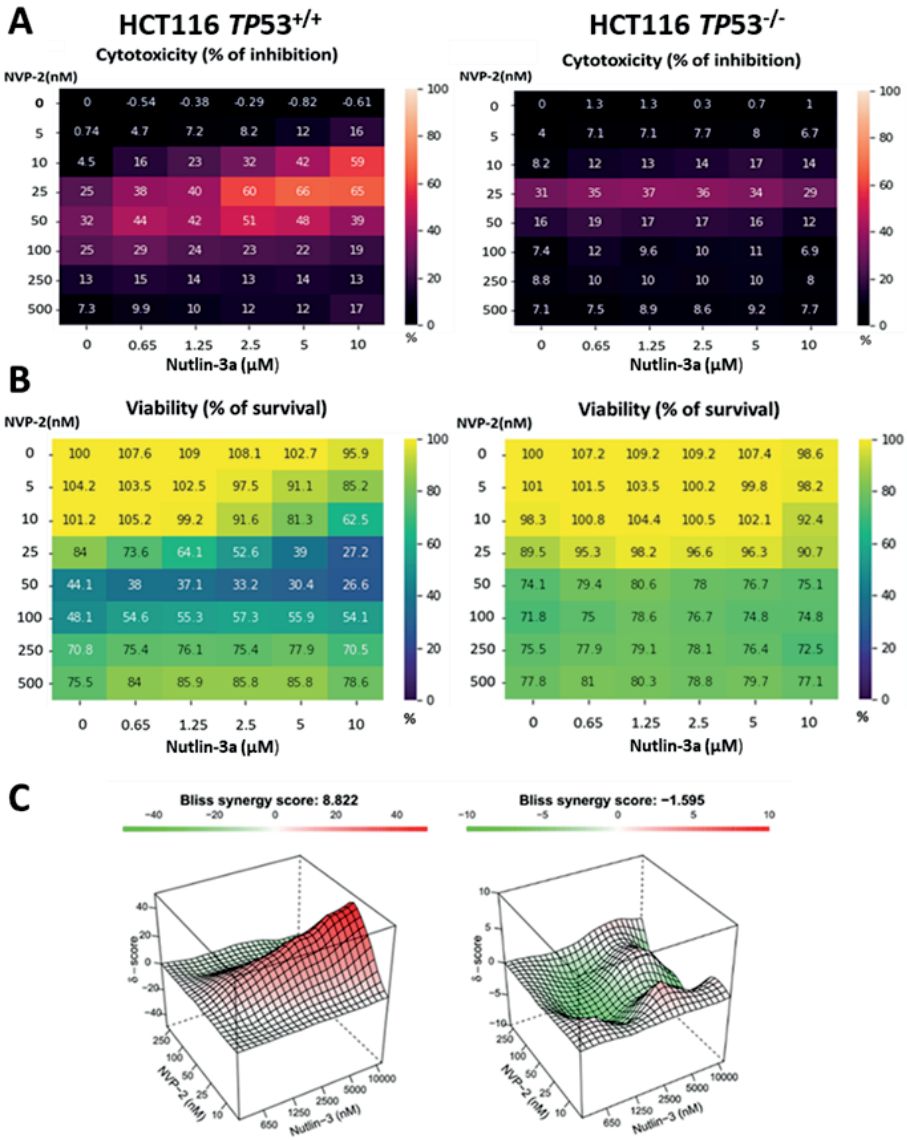

Figure 15. Activation of p53 is synthetic lethal with inhibition of CDK9 in HCT116 cells. A. HCT116 TP53$^{+/+}$ (left) TP53$^{-/-}$ (right) cells were treated with increasing concentrations of NVP-2 and Nutlin-3a and subjected for CellTox Green Cytotoxicity Assays (Promega) 48 h upon the treatment, average % of inhibition from three biological replicates. B. The same as A but for cell viability in CellTiter Glo 2 Assay (Promega). C. Synergy scores calculated using Bliss score with the assistance of SynergyFinder.

The above findings indicate that CDK9 activity is critical to the survival of cancer cells with activated p53. Co-treatment of the cells using p53-activating Nutlin-3a with a low concentration of CDK9 inhibitors leads to apoptosis in this cancer cell line. Besides, killing the cells with CDK9 inhibitors is dependent on the p53-driven apoptosis.

10.9. Synthetic lethality of pharmacological activators of the p53 pathway and CDK9 inhibitors relies on Pol II transcription

Next, we decided to study the mechanisms of observed synthetic lethality of p53-activators and CDK9 inhibitors in the cancer cell model. Given the role of CDK9 in promoting Pol II transcription elongation, we hypothesized that inhibition of the Pol II transcription in general during p53 activation might be the reason for observed cytotoxicity. To address this, we inhibited Pol II transcription upstream to P-TEFb using triptolide which inhibits the XBP subunit of TFIIH binding to Pol II disabling productive transcription initiation (Titov et al., 2011).

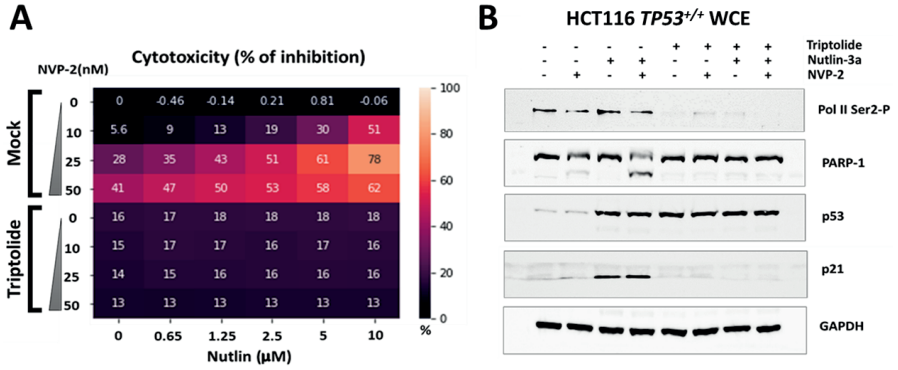

Figure 16. Effect of Pol II transcription inhibition on synthetic lethality in between NVP-2 and Nutlin-3a in HCT116 *TP53*$^{+/+}$ cells. A. Cytotoxicity in HCT116 *TP35*$^{+/+}$ cells in response to indicated drug treatments for 48 h in CTxG assay, average% of inhibition from three biological replicates shown. B. Immunoblot analysis of the whole-cell extracts treated with indicated combinations of 1 μM triptolide, 10 μM Nutlin-3a and 10 nM NVP-2 for 24 h. (Bugai A. et al. unpublished results).

We quantified cytotoxicity using the CTxG assay in HCT116 cells treated with Nutlin-3a and NVP-2 as in Figure 15A, or in combination with 1 μM triptolide. Notably, while triptolide had some residual cytotoxicity, inhibition of TFIIH completely eliminated the synergistic cytotoxicity effect of p53 activation and CDK9 inhibition (Figure 16A) (Bugai A. et al. unpublished results). Using immunoblot analysis of the whole-cell extracts we showed that triptolide does not attenuate the p53 stabilization by Nutlin-3a or NVP-2, but rather induced p53 protein levels by itself (Figure 16B). In contrast, triptolide efficiently decreased Pol II Ser2-P levels in cells, confirming the efficiency of Pol II transcription inhibition. Critically and in support of our CTxG data, PARP-1 cleavage in Nutlin-3a and NVP-2 co-treated cells was diminished by triptolide (Figure 16B). These results indicate that p53-dependent Pol II transcription, which does not rely on CDK9, is required for the observed synthetic lethality in HCT116 cells. In turn, CDK9-driven transcription is

essential for cell survival when the pro-apoptotic p53 transcription program is active. Taken together, our results provided new evidence that nongenotoxic activation of p53 triggers the dependency of the colon cancer cells on P-TEFb activity.

11 DISCUSSION AND FUTURE PROSPECTS

The present dissertation describes a novel mechanism controlling the Pol II transcription response to DNA damage via RBM7. In this section, I discuss the results in the context of the state of knowledge in the field and the prospects of its expansion through the presented research outcomes.

11.1. Identification of RBM7 as a novel P-TEFb release factor

Previous work identified RBM7 to promote the viability of cells during DDR, induced by UV light or 4-NQO. Here, the activation of the p38 MAPK pathway triggers phosphorylation of the serines 136 and 204 of RBM7 via MK2 kinase (Blasius et al., 2014). Indeed, the p38 MAPK pathway is connected to both DDR-induced RNA metabolism (Tiedje et al., 2015) and Pol II transcription rearrangements (Borisova et al., 2018). Upon DDR phosphorylated RBM7 stops binding to uaRNAs, leading to the accumulation of these short-lived transcripts in the cells (Blasius et al., 2014). However, it was unknown how exactly the loss of RBM7 could sensitize the cells to DNA damage.

In this research project, we hypothesized that RBM7 may bind additional RNAs upon its DDR-induced phosphorylation to mediate cell survival. Using iCLIP, we expanded the knowledge on DDR-induced RNA interactome of RBM7 (I: Figure 1) and found an unexpected link between RBM7 and 7SK, which in turn regulates the transcriptional response by Pol II. Namely, RBM7 serves as a P-TEFb release factor from 7SK snRNP (I: Figures 2-4) acting through the DDR-induced binding to its core 7SK snRNA. Our *in vitro* data suggests that direct phosphorylation-induced binding of RBM7 to LARP7 and MePCE may contribute to this effect (I: Figure 4E-G). Previously a highly stable trimeric NEXT complex, which consists of hMTR4 and ZCCH8 in addition to RBM7, was described (Lubas et al., 2015). The studies presented in this dissertation showed that, in contrast to RBM7, other NEXT subunits do not participate in the release of P-TEFb from 7SK snRNP (I: Figure S1-3). However, since the interaction of hMTR4 and ZCCH8 with 7SK snRNP was observed in whole-cell extracts (I: Figure 2), it is likely that RBM7 in living cells interacts with 7SK as part of NEXT. Several P-TEFb release factors were described in recent years (Table 1). For instance, RNA-helicase DDX21 (Calo et al., 2015) via its enzymatic activity on the 7SK is capable to release P-TEFb. RBM7, even in the absence of co-factor RNA-helicase hMTR4, is capable of remodeling 7SK snRNP (I: Figure 4C, D). This observation places RBM7 in

closer relationships with viral P-TEFb release factors, suggesting that RNA affinity, like in the case of HIV-1 Tat, plays an essential role in the mechanism involved (Garber et al., 1998; Krueger et al., 2010).

Of note, inhibition of the p38 MAPK pathway with the highly selective p38 inhibitor was enough to diminish the DDR-induced RBM7 binding to 7SK, P-TEFb release and DDR-genes accumulation (I: Figure 3C-D, S5D). This result indicates the upstream position of this pathway and the importance of phosphorylation of RBM7 for performing the activation of P-TEFb upon DNA damage. Importantly, this dissertation has for the first time linked an effector of the molecular pathway induced in response to DNA damage to P-TEFb release.

11.2. Defining of P-TEFb-dependent Pol II transcriptional program in response to DNA damage

Consequently, we examined if the DDR-induced P-TEFb release from the 7SK snRNP by RBM7 has an impact on the functions of P-TEFb in promoting gene transcription. Previously, many researchers have proposed that the P-TEFb release upon cell stress compensates for the global inhibition of transcription elongation (Chen et al., 2008; Napolitano et al., 2010; Rockx et al., 2000). At the same time, a growing body of genome-wide transcriptional data indicated the existence of DDR-induced Pol II transcription downstream to the promoter-proximal pause sites (Williamson et al., 2017). Active Pol II elongation upon DNA damage supports chromatin state, expression of the essential genes, and DNA repair (Gomes et al., 2006; Lavigne MD, 2017).

Here, by using a metabolic labeling technique for nascent RNA sequencing, we described Pol II transcription activation at the short transcriptional units in response to the 4-NQO-mediated genotoxic insult (I: Figure 5C). Therefore, we concluded that the decrease of Pol II processivity at the bodies of long genes rather than the promoter-proximal pause release is responsible for the UV-mediated inhibition of transcription elongation described before (Wilson et al., 2013). Importantly, activation of transcription at the promoter-proximal regions following UV irradiation was observed before (Lavigne MD, 2017; Williamson et al., 2017).

The p38 MAPK pathway had been suggested recently to be involved in the release of paused Pol II. Namely, the activation of the p38 MAPK induces the dissociation of NELF-E from the promoters of DDR genes enabling transcription elongation (Borisova et al., 2018). Of note, P-TEFb is known to phosphorylate NELF-E triggering its displacement from the paused Pol II complex (Fujinaga et al., 2004). Given our findings of RBM7-mediated P-

TEFb release from 7SK snRNP, RBM7 likely also participates in the process of NELF-E removal from the promoters.

In the current work, we show that the activity of P-TEFb is vital for the transcriptional activation at short transcription units, genes, and ncRNAs, essential for cell survival under genotoxic insult. Examples featured in this dissertation include genes encoding anti-apoptotic *MCL1* (Zhou et al., 1997), pro-survival *CDKN1A* (Napolitano et al., 2007) as well as the number of uaRNAs and eRNAs involved in the regulation of protein-coding genes in *trans* (Melo et al., 2013). In addition, the release of paused Pol II via the RBM7-P-TEFb axis could promote the sensing of DNA adduct sites by elongating Pol II and subsequent DNA repair (Gregersen and Svejstrup, 2018; Lavigne MD, 2017).

11.3. Activation of Pol II transcription of ncRNAs in response to 4-NQO

The results of nascent RNA sequencing suggest an important role of ncRNA transcription in the global gene transcription response to DNA damage (I: Figure 5B). Current work reports that in contrast to protein-coding genes, the clear majority of differentially expressed lncRNAs, uaRNAs and eRNAs showed uniform and P-TEFb-dependent transcription upregulation in response to 4-NQO treatment. Additional studies will give insights on whether the observed phenomenon has a functional impact. Of note, uaRNAs and eRNAs have been reported to rely on P-TEFb activity (Flynn et al., 2011). Though activation of uaRNAs and eRNAs may be a result of the rapid increase in chromatin accessibility around DDR genes (Sobhy et al., 2019; Younger and Rinn, 2017), these ncRNAs could support enhancer function of target genes or promoting transcription recovery by attenuating the expression of transcription repressors (Melo et al., 2013; Schaukowitch et al., 2014; Williamson et al., 2017). Finally, some of the activated RNAs may be involved in DDR directly, as functional lncRNAs (Liu et al., 2018; Schmitt et al., 2016).

Interestingly, 7SK was proposed previously to regulate bidirectional transcription at enhancers (Flynn et al., 2016). Inhibition of 7SK using antisense DNA-oligonucleotides induces the upregulation of the short-lived Pol II transcripts (Castelo-Branco et al., 2013), many of which are targets of nuclear exosome (Flynn et al., 2011). Here, activation of uaRNAs correlated weakly with the activation of their host protein-coding genes, suggesting that bidirectional transcription is involved as part of the transcriptional DDR (Figure 8). Additionally, eRNA linked to the DDR-responsive EGR2 gene showed similar transcription dynamics to the EGR2 gene transcript (I: Figure

5E) (Yokota et al., 2010). Thus, the results of this work indirectly support a vital role of transcriptional activity at promoter-proximal elements and enhancers in regulating the gene expression (Kaikkonen and Adelman, 2018). In prospect, DDR-induced ncRNAs accumulation is a perspective model to study these relationships (Hawley et al., 2017).

11.4. Inhibition of P-TEFb within 7SK snRNP as an essential step of the regulation of Pol II transcription

In agreement with recent work, this thesis confirms the complex regulatory role of the 7SK snRNP complex in transcription (McNamara et al., 2013; McNamara et al., 2016). 7SK snRNP serves as a reservoir of suppressed P-TEFb which could be activated through P-TEFb release factors to achieve rapid promoter-proximal pause release. Such a system likely is not limited to DDR and acts in other cell-stimulating conditions (Table 1). In addition, the establishment of stable promoter-proximal pausing of Pol II may be dependent on 7SK (Bacon and D'Orso, 2018). In the absence of 7SK, the possibility of fast transcriptional response upon appropriate stimuli is corrupted and P-TEFb prematurely targets the paused Pol II complexes release polymerases into the bodies of the genes.

Our data (I: Figure 6D) suggests that repression of P-TEFb within 7SK snRNP is essential for rapid Pol II activation. The additional mechanistic studies will address whether, following DDR, P-TEFb is recruited to target gene promoters in the inhibited form within 7SK snRNP or P-TEFb release takes place outside of chromatin. Interestingly, KRAB-interacting protein 1 (KAP1) (Iyengar and Farnham, 2011) was described to play a critical role in tethering 7SK snRNP to the promoters of the inducible genes (McNamara et al., 2016). Moreover, DNA damage was shown to stimulate the chromatin recruitment of KAP1 through ATM-dependent phosphorylation of KAP1 (Ziv et al., 2006). Further research is needed to find out whether KAP1 brings 7SK snRNP also to DDR-induced promoters to facilitate additional control of their expression.

11.5. The role of DNA-binding TFs in the regulation of the P-TEFb-driven transcription program

The work highlights the importance of DNA-binding TFs for directing the released pool of P-TEFb in the wake of genotoxic insult to select target

chromatin regions. Massive P-TEFb activation (I: Figure 2F, 4A) by RBM7 after DNA damage could not explain the high specificity of the transcriptional response (I: Figure 5A). The TFs, which capture released P-TEFb, should provide specificity to the transcriptional induction by Pol II (I: Figure 7E). By analyzing the 4-sU sequencing data, we identified many TFs that could play a role in this process, such as NF-κB, p53, ATF4, FOS, JUN and others (Yan et al., 2007) (I: Figure S3C, Table S3D).

One of the most important players in DDR, which is likely involved directly or indirectly in utilizing the active P-TEFb following genotoxic insult, is tumor suppressor protein p53 (Radhakrishnan et al., 2008). Of note, P-TEFb was described previously as essential for the p53 functions, since many of p53 target genes depend on P-TEFb activity towards Pol II (Gomes et al., 2006; Sullivan et al., 2018; Wang et al., 2014). My yet unpublished data (Figures 12-15) suggest that P-TEFb is involved in the p53 transcriptional program even in the absence of DNA damage. Interestingly, the Pol II inhibitor triptolide blocked the synthetic lethality between p53 activation and P-TEFb inhibition (Figure 15) showing that the p53-mediated apoptotic cell death is driven by Pol II transcription (Albert et al., 2016).

11.6. Opportunities for inhibition of the P-TEFb-mediated transcriptional DDR in cancer treatment

Many cancers depend on P-TEFb-driven transcription *per se* because of the constant need for the expression of key oncogenes (Bradner et al., 2017; Franco et al., 2018; Morales and Giordano, 2016). Of note, pan-CDK inhibitors such as flavopiridol or dinaciclib are efficient drivers of apoptosis, for instance, in acute myeloid leukemia cells both *in vitro* and *in vivo* (Chen et al., 2016; Gregory et al., 2015; Zhang et al., 2017). In contrast, during the clinical trials, flavopiridol and dinaciclib showed high rates of adverse effects, likely due to lack of selectivity (Gojo et al., 2013; Lyle and Daver, 2018; Morales and Giordano, 2016). Also, many recent studies focus on the direct role of CDK9 transcription control in cancer and the possibilities of pharmaceutical perturbations for therapy (Boffo et al., 2018; Moreno et al., 2017; Rahaman et al., 2018; Yin et al., 2014). A new generation of specific CDK9 inhibitors, such as NVP-2 or i-CDK9 (Lu et al., 2015; Olson et al., 2018), is expanding the possibilities to dissect molecular pathways controlled by this kinase in cancer and to find new ways to target CDK9 functions in a therapy setting.

Our results presented in my dissertation suggest an opportunity to drive apoptosis in cancer cells by P-TEFb inhibition in combination with DDR induction by chemotherapy agents (Figures 12, 15). Therefore, the current

application of CDK9 inhibitors may be enhanced via the proposed model of induced transcription dependency (Kalan et al., 2017) (Figure 17). Of note, cancer cells rely on DDR pathways to resist chemotherapy treatment (Jackson and Helleday, 2016). Since inhibition of P-TEFb counteracts the pro-survival Pol II transcriptional response to DNA damage, DDR-inducing chemotherapeutic agents may serve as inducers of transcriptional dependency to CDK9 (Dasari and Tchounwou, 2014; Heidelberger et al., 1957).

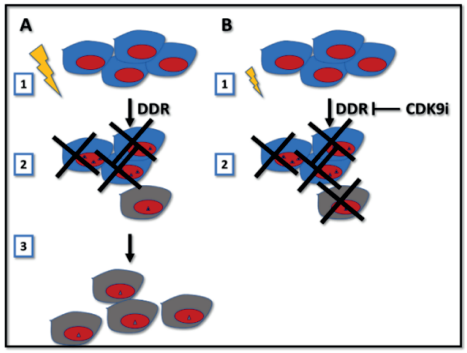

Figure 17. Schematic representation of the proposed strategy. A. Cancer cells treated with high doses of DNA damaging agent (1) exhibit DDR and are eliminated by apoptosis because of the loss of genomic integrity (2), while a small fraction of cells survive and provide the basis of resistance to the treatment (3). B. Cancer cells treated with moderate doses of DNA damage agent, just enough to induce DDR (1), which makes them dependent on CDK9. Simultaneous pharmacological inhibition of CDK9 blocks the pro-survival part of DDR and the appearance of resistant cells (2).

In addition to DDR induction (I: Figure 7), my thesis further shows the possibility to induce cancer cell dependency on CDK9 transcription through nongenotoxic means (Figure 15). Because such a nongenotoxic case of dependency on P-TEFb alleviates the risk of mutagenesis by DNA damaging chemotherapeutics, extending our experiments with the p53 activating drug Nutlin-3a should spur further interest in the field. Since there are a number of widely used DDR-inducing chemotherapy agents (Chuang et al., 2012; Kuo et al., 2016; Stubbert et al., 2010), and additional p53 inducers (di Iasio and Zauli, 2013; Kojima et al., 2006), translation of our results to appropriate cancer models with wild-type *TP53* will serve as a tempting direction for future research.

12 CONCLUSIONS

The thesis reveals several new insights of transcription regulation within the cellular response to genotoxic stress:

- Firstly, using the high-throughput RNA-interactome and mechanistic studies I discovered a new function of RBM7. RBM7 is an essential effector of the DDR signaling network, which is orchestrated by the p38 MAPK kinase. RBM7 releases P-TEFb from the inhibitory 7SK snRNP in response to DDR, which relies on DDR-induced interaction of RBM7 with 7SK.

- Secondly, my work defines a central role of P-TEFb, released from 7SK snRNP, in the transcriptional response to DNA damage. P-TEFb is vital for triggering the Pol II release from promoter-proximal pausing and expression of DDR genes. Notably, RBM7 is necessary for P-TEFb release and cell survival upon genotoxic insult.

- Thirdly, since P-TEFb activation upon DNA damage is global, cellular TFs that capture P-TEFb on chromatin direct the Pol II transcription locally. Interestingly, p53, the critical DDR-induced transcription factor, is involved in P-TEFb-dependent transcription.

- Finally, P-TEFb deficiency renders cancer cells highly sensitive to DDR-activating compounds and p53 activation. Hence, I propose that targeting of the P-TEFb-dependent pro-survival arm of DDR is a tempting opportunity for novel anti-cancer strategies.

13 ACKNOWLEDGMENTS

The work was performed in the Center for Gene Regulation at the Department of Biochemistry and Developmental Biology, Faculty of Medicine, University of Helsinki. The research work and training were supported by the Academy of Finland, University of Helsinki, Sigrid Juselius Foundation, Doctoral Program of Biomedicine, K. Albin Johanssons Stiftelse, Maud Kuistila Memorial Foundation.

First, I wish to thank my supervisors. Matjaž Barborič provided generous support and the opportunity to work on this exciting topic, excellent supervision, help with the research work throughout these years and the revision of the thesis. I am grateful to Dan Lindholm, who was supervising the progress of the thesis from the very beginning and provided support and guidance along this way, for vivid discussions and the critical revision of the current manuscript.

I was happy to get such thesis committee members, as Sampsa Hautaniemi and Mikko Frilander, who provided advices and roadmap for converting the science into my degree. It was essential to get the opinion from the outside of the lab to move the research forward and set up realistic and clear goals for the research project. My external examiners, Lea Sistonen and Minna Kaikkonen-Määttä, helped me to improve this dissertation significantly by the critical revision of the thesis manuscript, interesting suggestions and the feedback, which made me rethink several aspects of my work. I thank Robert Fisher for agreeing to serve as an opponent for this dissertation and looking forward to the interesting discussion, which will be it for sure.

I thank previous and current members of the laboratory, with whom I collaborated during all these years: Alexandre Quaresma, Tina Lenasi, Zhijia Wang, Petra Kukanja, Neja Sirc and Kingsley Ekumi for teaching me new techniques, interesting research work and healthy environment, which helped me to achieve my goals. I had the luck to work with prominent colleagues, involved in the project, and co-authoring the publications. Lars Dölken, Thomas Hennig, Caroline Friedel, Christopher Sibley, Jernej Ule, Koh Fujinaga, Robert Düster, Matthias Geyer were involved in the research work of this thesis and played an essential role in the success of the project.

I am thankful to the members of the Biomedicum community who made the environment in which I managed to survive. Reagent requests or moral support, there were always people around. Members of the Optobiology Lab were the best neighbors during the last years. I am grateful to meet Taras Redchuk, whose friendship and support was always there when needed. I wish to thank Denis Belitskin for that antibody I was looking during revision, as well as for nice beer during happy hours. Tarja Grundström helped me to find out

how to run a laboratory business and how to find things at the department during the research project. Valentyn Oksenych provided reasonable career advice, moral support, and friendship during these long years of my Ph.D. I am grateful to Denis Kainov for the interesting collaborative work, friendship, and support he provided to me. My friends back from high school and Kharkiv University time were with me all the way, and even remotely, their support motivated me and gave me the opportunity of being happy and keep going.

I want to express gratitude to the members of my family. My family gave me the motivation and support critical for the completion of this thesis and preserving of my physical and mental health. My parents Yurii Buhai and Olha Buhai raised me, my passion for science and biology, and my ambitions to do the best I could, gave me the possibility to develop in the hard times of political instability. I thank my sister, Oksana Buhai, with whom I shared my childhood. I am happy to have a grandmother, Halyna Polunina, who is interested in my work and the world around. I thank my grandmother, Pasha, my grandfathers, Viktor and Vladimir, who haven't made it to this moment, for my happy childhood, which shaped my personality. I am grateful to my beloved wife, Anhelina, for being my love, my friend, and my team player, allowing me to gain more.

Finally, I am thankful to the members of the public who read this thesis to this end and hope it changed something in them.

Helsinki, 2020

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